DOCTORAL THESIS

Psychological Stress and Bladder Dysfunction.

West, Eliza

Award date:
2021

Psychological Stress and Bladder Dysfunction

Eliza Grace West

Submitted in total fulfilment of the requirements of the degree of

Doctor of Philosophy by Research

January 2021

Faculty of Health Sciences and Medicine

Associate Professor Catherine McDermott, Associate Professor Donna Sellers and Professor Russ Chess-Williams

The research was support by an Australian Government Research Training Program Scholarship.

ABSTRACT

This thesis investigates the effects of psychological stress on bladder function in mice and the potential benefit of current clinical therapies. Psychological stress and bladder dysfunction encompass a wide range of disorders known to affect a large portion of the society. There is convincing clinical evidence that links psychological stress with bladder dysfunction, but the underlying physiological mechanisms involved, and suitability of drug treatments, remain elusive. The effects of social stress, witness stress and environmental stress on bladder function were observed using a social defeat and witness trauma model, as well as a water avoidance stress model, with animals exposed to stress for 1 h/day for 10 consecutive days. These models were used to investigate the effects on voiding behaviour and local bladder function.

Social defeat but not witness trauma stress decreased urinary frequency in male mice, indicative of urinary retention, which could be due to the increase in purinergic responses observed in the isolated whole bladder preparations from these mice. In contrast, the water avoidance model produced increased urinary frequency in female mice and an increase in overall contractility of the bladder. Due to the clinical association between psychological stress and overactive bladder (OAB), rather than urinary retention, recovery studies were completed in the water avoidance stress model, as were drug treatment studies using the anxiolytic sertraline and commonly used OAB drugs mirabegron and solifenacin.

Ten-days stress-free recovery increased bladder compliance in water avoidance stressed animals and reduced urinary frequency, although not back to unstressed control levels, with a new elevated baseline evident. All drug treatments decreased the hormonal response to stress, measured as plasma corticosterone levels, and decreased voiding dysfunction, measured as voiding frequency. Sertraline, however, reduced stress

hormone levels the most, while also targeting the increased bladder contractility observed after psychological stress more efficiently. Despite this, mirabegron and solifenacin were more effective at reducing the impact of stress on urinary frequency, returning voiding behaviour to that of unstressed control animals.

Overall, bladder dysfunction induced by stress exposure appears to be dependent upon sex and/or stressor type; with urinary retention evident in male mice following social defeat, no voiding changes in male witness mice and development of an overactive phenotype in female mice after water avoidance stress. The bladder overactivity induced by water avoidance stress was associated with detrusor hypercontractility. Mirabegron and solifenacin were equally effective at reducing the impact of water avoidance stress on voiding behaviour and were both superior to the selective serotonin reuptake inhibitor sertraline.

DECLARATION

This thesis is submitted to Bond University in fulfilment of the requirements of the degree of Doctor of Philosophy by Research.

This research represents my own original work towards this research degree and contains no material which has been previously submitted for a degree or diploma at this university or any other institution, except where due acknowledgment has been made.

Eliza Grace West

08/01/2021

RESEARCH OUTPUTS AND PUBLICATIONS DURING CANDIDATURE

Abstracts as a result of this thesis

"The anxiolytic sertraline prevents the effects of psychological stress on voiding behaviour and contractile bladder responses" Eliza West, Donna Sellers, Catherine McDermott and Russ Chess-Williams, *29th National Conference on Incontinence*, Brisbane and remote, 18th-21st November 2020.

"Mirabegron and solifenacin reduce voiding dysfunction caused by psychological stress" Eliza West**,** Donna Sellers, Catherine McDermott and Russ Chess-Williams, 29th National Conference on Incontinence, Brisbane and remote, 18th-21st November 2020.

"Social defeat and witness trauma is associated with increased corticosterone levels and enhanced contractile bladder responses" **Eliza West,** Donna Sellers, Catherine McDermott and Russ Chess-Williams, *49th Annual Meeting of the International Continence Society*, Gothenburg 3rd-5th September 2019

"Mechanisms of bladder dysfunction are dependent on type of psychological stressor" **Eliza West**, Donna Sellers, Catherine McDermott and Russ Chess-Williams, 11th *National Symposium on Advances in Urogenital and Gut Research*, 2nd September 2019

"Water avoidance stress induced bladder overactivity in mice is associated with enhanced contractile bladder responses" **Eliza West**, Donna Sellers, Catherine McDermott and Russ Chess-Williams, 10th *National Symposium on Advances in Urogenital and Gut Research*, 26th November 2018

"Stress causes bladder overactivity in mice: enhanced voiding and detrusor contractility" **Eliza West**, Donna Sellers, Catherine McDermott and Russ Chess-Williams, *27th National Conference on Incontinence, Hobart, Tasmania*, 24th-27th October 2018

"Water avoidance stress induced bladder overactivity in mice is associated with enhanced contractile bladder responses" **Eliza West**, Russ Chess-Williams, Donna Sellars and Catherine McDermott, *48th Annual Meeting of the International Continence Society*, Philadelphia 28th-31st August 2018

Journal publications as a result of this thesis

"Bladder overactivity induced by psychological stress in female mice is associated with enhanced bladder contractility" **E.G. West**, D.J. Sellers, R. Chess-Williams and C. McDermott. *Life Sciences* (2020) https://doi.org/10.1016/j.lfs.2020.118735

"Voiding behaviour and efferent bladder function altered in mice following social defeat but not witness trauma" **Eliza G West**, Donna Sellers, Russ Chess-Williams and Catherine McDermott (2020) *Frontiers in Physiology* 11(247); (Impact Factor 4.1)

"The anxiolytic sertraline reduces the impact of psychological stress on bladder function in mice" **West EG**, Sellers DJ, Chess-Williams R, and McDermott C. *Life Sciences* (2021) https://doi.org/10.1016/j.lfs.2021.119598

ETHICS DECLARATION

The research associated with this thesis received ethics approval from the University of Queensland Animal Research Ethics Committee. Ethics application number - BOND/536/17.

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The citations of each work also appear in the preface of the relevant chapters within the thesis.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my supervisor's Associate Professor Catherine McDermott, Associate Professor Donna Sellers, and Professor Russ Chess-Williams for giving me the opportunity to complete this PhD and for their ongoing support and encouragement. Their enthusiasm and passion for research inspired me throughout my three years, especially during the twelve-hour lab days. It has been an absolute pleasure to work with and learn from them throughout this process and I could not imagine taking on this endeavour without them as my mentors.

I would also like to acknowledge the funding granted by the Australian Bladder Foundation throughout my PhD. I also thank Bond University and the faculty of Health Sciences and Medicine who have provided support through this PhD, and all the additional staff who have helped me get to where I am today. Thank you to the Centre for Urology Research, both staff and students, for our weekly meetings which motivated me throughout these three years. Marion Andreas and Katie Powell, without you both the lab space would not function, and I am beyond appreciative of the time and effort you put into helping me through my experiments. I would also like to thank the HDR students who have been by my side for the late nights and early mornings. Our office is the way it is because of all of you, and I could not have asked for a better group of people with whom I have shared this experience.

Of course, I have to thank all of my friends, especially Julia Shirley, Soni Yadav, Dominique Jones and Keegan Scerri, who have been a part of this experience. Zoe Porter, from the undergraduate Biomedical Science days to our PhD journeys, you have shown me endless support and I cannot describe what it means to have you as a friend. Thank you to all my other friends, who encouraged me during these years, and gave me the 'between times.' The times that were not necessarily the PhD but those that made the journey easier.

Thank you to my biggest cheerleader, twin sister and best friend, Eleanor West, for getting me through, not just the PhD, but life as well. Thank you to Nan (Joyce Bambach), who was the best house mate, and Grandmother I could ask for.

Finally, thank you to Mum and Dad (Vanessa and Andrew West). There is not enough space here or words to describe what their support has meant to me over the past three years. At the end of senior school, six years ago, they encouraged me to apply to Bond and now I'm completing my PhD. This thesis is dedicated to you both. Don't worry, I'll never make you read it, we'll just wait for the movie version to come out.

Table of Contents

ABBREVIATIONS

αβmATP: alpha, beta-methylene adenosine-triphosphate
ACh: Acetylcholine
ACTH: Adrenocorticotropic hormone
AChR: Acetylcholine Receptor
AR: Adrenergic receptor
ATP: Adenosine-triphosphate
ATPase: Adenosine-triphosphatase
AVP: Arginine vasopressin
BDNF: Brain derived neurotropic factor
BPH: Benign Prostatic Hyperplasia
Ca^{2+}: Calcium
CaM: Calmodulin
cAMP: Cyclic AMP
CarAT: carnitine acetyltransferase
cGMP: Guanosine 3',5'–cyclic monophosphate
ChAT: Choline acetyltransferase
CHT1: Choline transporters
CNS: Central nervous system
COX-2: Cyclo-oxygenase 2
CRF: Corticotrophin releasing factor
CRH: Corticotropin releasing hormone
DAG: Diacylglycerol
EFS: Electrical field stimulation
eNOS: Endothelial nitric oxide synthase
EuroQoL: European Quality of Life
FST: Forced Swim Test
GABA: Gamma-aminobutyric acid
GDP: Guanine di-phosphate
GI: Gastrointestinal
GRs: Glucocorticoid receptors
HPA: Hypothalamic-pituitary-adrenal
HRP: Horse radish peroxidase
HRQoL: Health Related Quality of Life
H_2O_2: Hydrogen peroxide
IC: Interstitial Cells
KCl: Potassium chloride
KO: Knockout
L-NNA: N_ω-nitro-L-arginine
LUT: Lower urinary tract
mmHg: millimetre of mercury
mRNA: Messenger ribonucleic acid
IC: Interstitial cystitis
iNOS: Nitric Oxide Synthase
IP_3: Inositol triphosphate
MLC: Myosin light chain
MRs: Mineralocorticoid receptors
NA: Noradrenaline
Na^{2+}: Sodium
NANC: non-adrenergic, non-cholinergic
NO: Nitric Oxide
NSB: Non-specific binding
OAB: Overactive Bladder Syndrome
OCT: Organic Cation Transporter
PBS: Painful bladder syndrome
PG: Prostaglandin
PIP: Membrane Phospholipids
PLC: Phospholipase C
PMC: Pontine micturition centre
PTSD: Post traumatic stress disorder
QoL: Quality of Life
ROCK: Rho-Kinase
SNRI: Serotonin-Norepinephrine Reuptake Inhibitor
SR: Sarcoplasmic Reticulum
SSRI: Selective Serotonin Reuptake Inhibitor
TCA: Tri-cyclic Antidepressants
TLRs: Toll-like receptors
TMB: Tetramethyl benzidine
TRP: Transient receptor potential channels
TTX: Tetrodotoxin
UDIF: Urothelial Derived Inhibitory
VaChT: Vesicular acetylcholine Transporter Factor
VPA: Voiding pattern analysis
VTA: Ventral tegmental area
WAS: Water avoidance stress

CHAPTER 1: INTRODUCTION

SIGNIFICANCE

Psychological stress and bladder dysfunction encompass a range of disorders which affect a large proportion of society. Stressors cannot generally be defined by any one cause and greatly depend on a person's surroundings, however the most common forms of psychological stress include anxiety and depression (Schneiderman, Ironson, & Siegel, 2005). A national survey by The Australian Institute of Health and Welfare has reported that 45% of the population were affected by a mental health disorder at some point in their lives. Of this, anxiety disorder was most prevalent, affecting 14.4%, and depression the next most common, afflicting 6.2% of this group (Statistics, 2008).

Several studies have observed links between psychological stress and disease outcomes. It is well understood that psychological stress often occurs as a result of disease, however, there have been studies which have associated psychological stress with the cause of disease/disorder. Many studies have observed that stress can greatly influence the development of bladder symptoms, or worsen symptom severity (Chang et al., 2009). In spite of this, very little research has focused on the precise changes and underlying mechanisms of psychological stress-induced bladder dysfunction.

Lower urinary tract symptoms (LUTS) are common among Australian men over the age of 45, with the most common symptoms being nocturia and urgency. Risk factors of LUTS include increased plasma glucose and abdominal fat as well as, low HDL cholesterol and obstructive sleep apnoea (Martin et al., 2011). Most men with LUTS have associated benign prostatic hyperplasia, which may make diagnosis difficult to distinguish from overactive bladder (Woo, Gillman, Gardiner, Marshall, & Lynch, 2011).

Overactive bladder, incontinence and interstitial cystitis are well documented forms of bladder dysfunction and have been widely researched over a number of years (Wallace & Drake, 2015). In 2020 the Continence Foundation of Australia reported that 10% of

Australian men and 38% of Australian women live with urinary incontinence and only 30% of the patients sought treatment from a health professional. The total financial cost of incontinence in 2010 was $271 million and was projected to rise to $450 million by this year, 2020 (Deloitte Access Economics, 2011).

The symptoms of bladder dysfunction have been shown to increase with age and are more frequently observed in women (Coyne et al., 2011). While not well understood, approximately 40% of patients with bladder dysfunction still report poor treatment outcomes from the currently available treatments (Teleman et al., 2004). This lack of effective treatment leads to declines in quality of life and mental health issues in patients, which may impact severity of psychological stress (Rothrock, Lutgendorf, Hoffman, & Kreder, 2002). Despite strong clinical associations, and reports of increased bladder symptom severity with stress, there has been very little attention given to the mechanisms underlying bladder dysfunction induced by stress and anxiety.

Thus, this thesis will further investigate the pathophysiological pathways and increase knowledge of the mechanisms causing changes in lower urinary tract (LUT) function following stress. Evidence will also be provided as to the most appropriate and beneficial pharmacotherapies for use in patients with urological dysfunction associated with chronic psychological stress.

THE BLADDER

Anatomy of the Bladder

The urinary bladder is a muscular sac which serves to collect, store and transport urine. The bladder lies posterior to the pubic symphysis, and anterior to the vagina and uterus of females, and the rectum in males (Hickling, Sun, & Wu, 2015). The bladder consists of several structures including the openings of the ureters and urethra, bladder trigone and a layered bladder wall. The layers of the bladder wall, from inside out, are the transitional epithelium (urothelium), lamina propria, detrusor muscle and adventitia **(Figure 1.1)** (Keane & O'Sullivan, 2000).

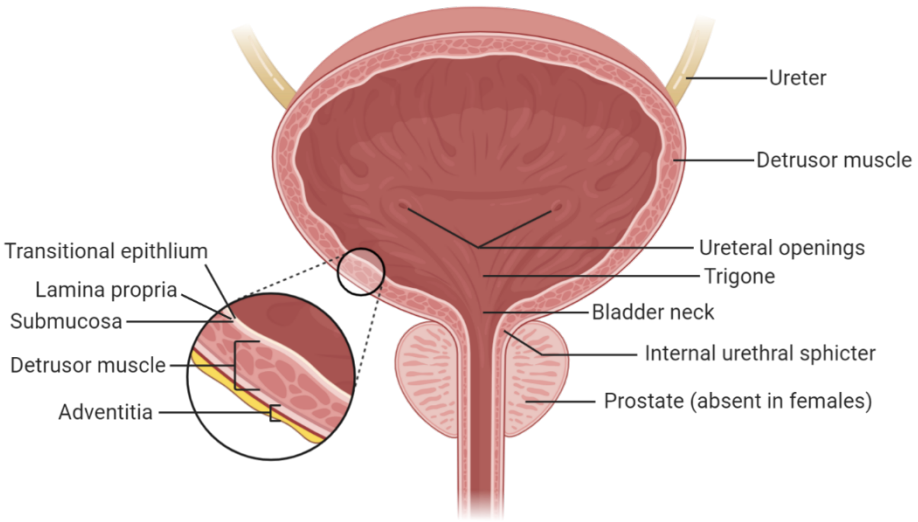

Figure 1.1: Diagram displaying the full bladder anatomy with detailed description of the bladder wall (Created with BioRender.com by the author).

The bladder urothelium is a continuous superficial lining, made up of several different cell types **(Figure 1.2.A)** (Hill, 2015). The three different cell types making up the urothelium include basal cells, intermediate cells and umbrella cells **(Figure 1.2.B)** (Lewis, 2000). Undifferentiated basal cells line the basement membrane and were originally thought to replenish damaged surface umbrella cells, however, studies have now found that intermediate cells contain the progenitor population used to repopulate

lost umbrella cells (Gandhi et al., 2013). The umbrella cells (top cell layer of the urothelium) are connected via tight junction, which produces an impermeable superficial layer of the bladder (Hill, 2015). The urothelium also serves a sensory and signalling role which will be discussed in detail later.

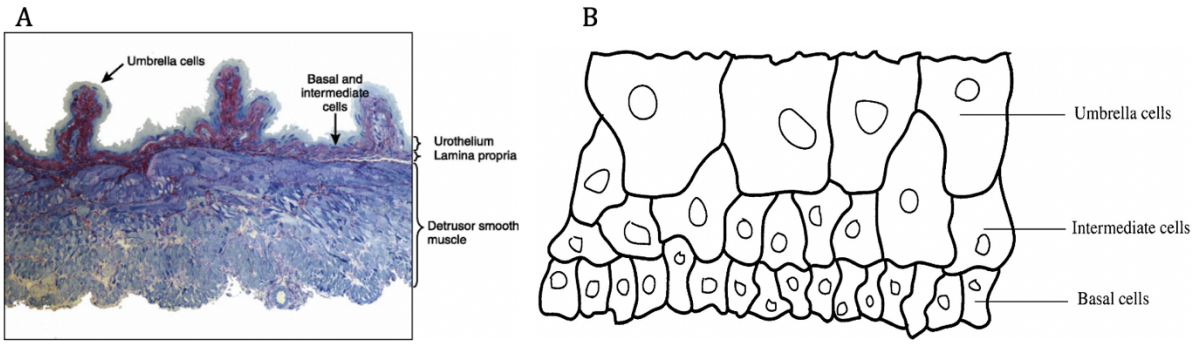

Figure 1.2: A. Histology of the murine bladder wall (Reproduced with permission from Hill (2015)) and B. Schematic drawing of cells of the bladder urothelium (Reproduced with permission from Lewis (2000)).

The lamina propria sits beneath the urothelium, and contains blood vessels, nerves, connective tissue and interstitial cells (IC) (Paner et al., 2007). While there has been some speculation as to the function of the ICs in the lamina propria, the most common theory is that the IC pace the smooth muscle bladder contractions during the filling phase of micturition and act as intermediates between the urothelium, nerves and muscle (Andersson & McCloskey, 2014). This theory is further strengthened by unpublished data from Monaghan & McCloskey, presented in Andersson and McCloskey (2014), collected on the mucosa-intact tissue strips from neurogenic rat bladder, which were found to develop better spontaneous contractions, compared to the controls.

The muscular layer called the detrusor is the thickest layer of the bladder wall. The smooth muscle contains a high number of receptors and signalling pathways, all of which are important for overall bladder function (Andersson & Arner, 2004). A non-linear

relationship is present between muscle stretch and tension eluding to the "stretch-relaxation phenomenon", meaning that the smooth muscle cells stretch and elongate to accommodate bladder filling (Orabi et al., 2013). During voiding, the cells shorten and generate the contractions required to empty the bladder through the urethra (Drew & Murphy, 1997). The urethra is lined by uroepithelial cells which express microvilli on the apical surface. The importance of this microvilli has not yet been determined, however, it has been considered to affect bacterial adherence (Birder & Andersson, 2013). There is evidence that the uroepithelial may also serve as sensors for mechanical and chemical signals, which play an integral role in regulation of the lower urinary tract (LUT) function (Snellings, Yoo, & Grill, 2012).

Innervation and Neurotransmission of the Bladder

The storage and periodic elimination of urine, from the bladder, is controlled by a multitude of neural circuits (Fowler, Griffiths, & de Groat, 2008). Complex interactions between the somatic and autonomic pathways are required to relax and contract the bladder during filling and voiding (Yoshimura & Chancellor, 2003). Micturition relies on both the urinary bladder as a reservoir and the bladder neck, urethra and urethral sphincter as an outlet (Fowler et al., 2008). The LUT is innervated by 3 sets of peripheral nerves, which include the pelvic parasympathetic nerves arising from the sacral level, the sympathetic nerves and the pudenal nerves **(Figure 1.3)** (Yoshimura & Chancellor, 2003).

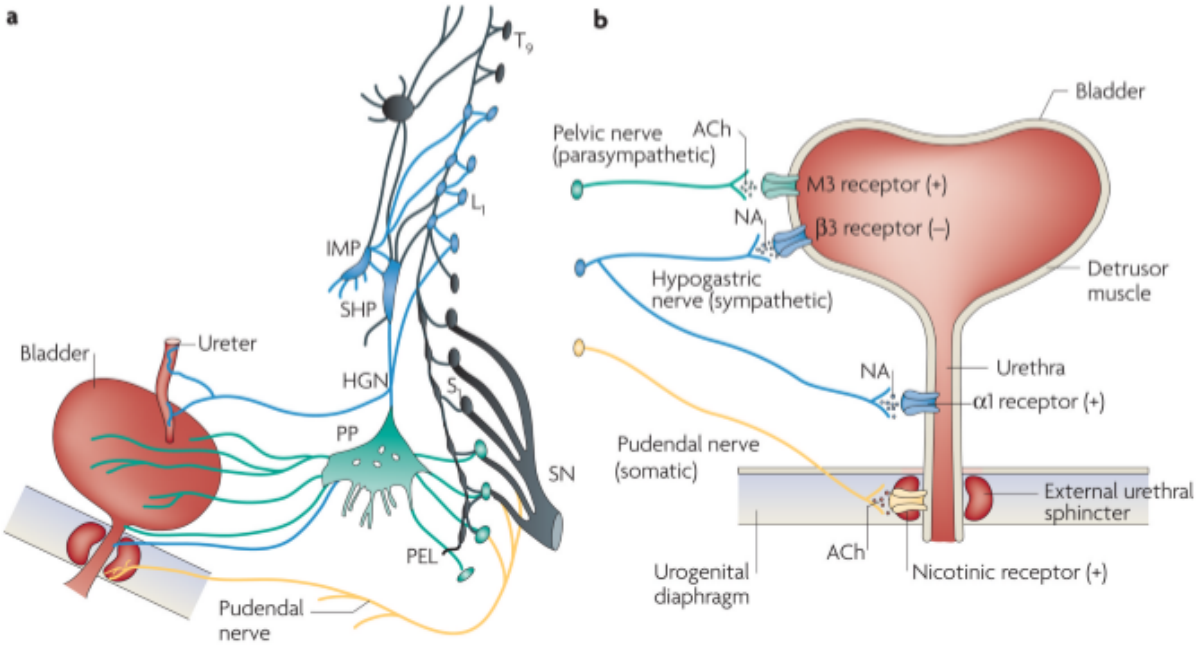

Figure 1.3: Innervation of the bladder. (A) Sympathetic fibres (blue), parasympathetic fibres (green) and somatic nerves (yellow); Hypogastric nerves (HGN), Inferior mesenteric plexus (IMP), Pelvic plexus (PP), Pelvic nerves (PEL), Superior hypogastric plexus (SHP). (B) Neurotransmitter release onto receptor types; Acetylcholine (ACh), Adrenergic receptors (α1 and β3), Muscarinic receptors (M_3), Noradrenaline (NA). (Reproduced with permission from Fowler et al. (2008)).

Sympathetic Innervation

The sympathetic innervation originates from the T11-L2 segment of the spinal cord. This runs through the mesenteric ganglia and hypogastric nerve to the bladder. The post-ganglionic nerves release noradrenaline (NA) to activate the β3-adrenoceptors (Yoshimura & Chancellor, 2003). This is responsible for the inhibition of the bladder smooth muscle, thereby keeping the bladder relaxed during filling.

β-adrenoceptors are members of the G protein-coupled receptor family and have 7 transmembrane spanning regions. They are classified as β_1-, β_2-, or β_3-adrenoceptors, with the main subtype in humans being β_3-adrenoceptor **(Figure 1.4)** (Li, De Godoy, & Rattan, 2004). β_1 and β_3-adrenoceptors have been observed to activate $G_{i/o}$-protein subunit, which subsequently goes on to stimulate the guanylate cyclase enzyme,

increasing guanosine 3'5'-cyclic monophosphate (cGMP) production **(Figure 1.4)**. The β_2-adrenoceptors, however, activate both $G_{i/o}$ as well as G_s-protein subunits which stimulate cGMP and adenylate cyclase (cAMP) respectively (Li et al., 2004). These second messengers result in relaxation in the smooth muscle.

Mirabegron, a drug which will be discussed in future chapters, acts as a β_3-adrenoreceptor agonist. The mode of action of the agonist is similar to NA, stimulating the receptors to increase relaxation of the detrusor smooth muscle, thereby increasing the volume of urine storage.

NA has also been noted to act on α_1-adrenoreceptors including subtypes; α_{1A}, α_{1B}, and α_{1D}, where α_{1A}- is the main subtype found in human urethra (Burnstock, 2007). α_1-adrenoreceptors are linked to the G_q-protein and when stimulated with activate enzyme PLC (not shown in Figure 1.4) and cause production of IP_3 **(Figure 1.4)**. This causes contraction and closure of the bladder neck during filling (Fowler et al., 2008; Hegde et al., 1998).

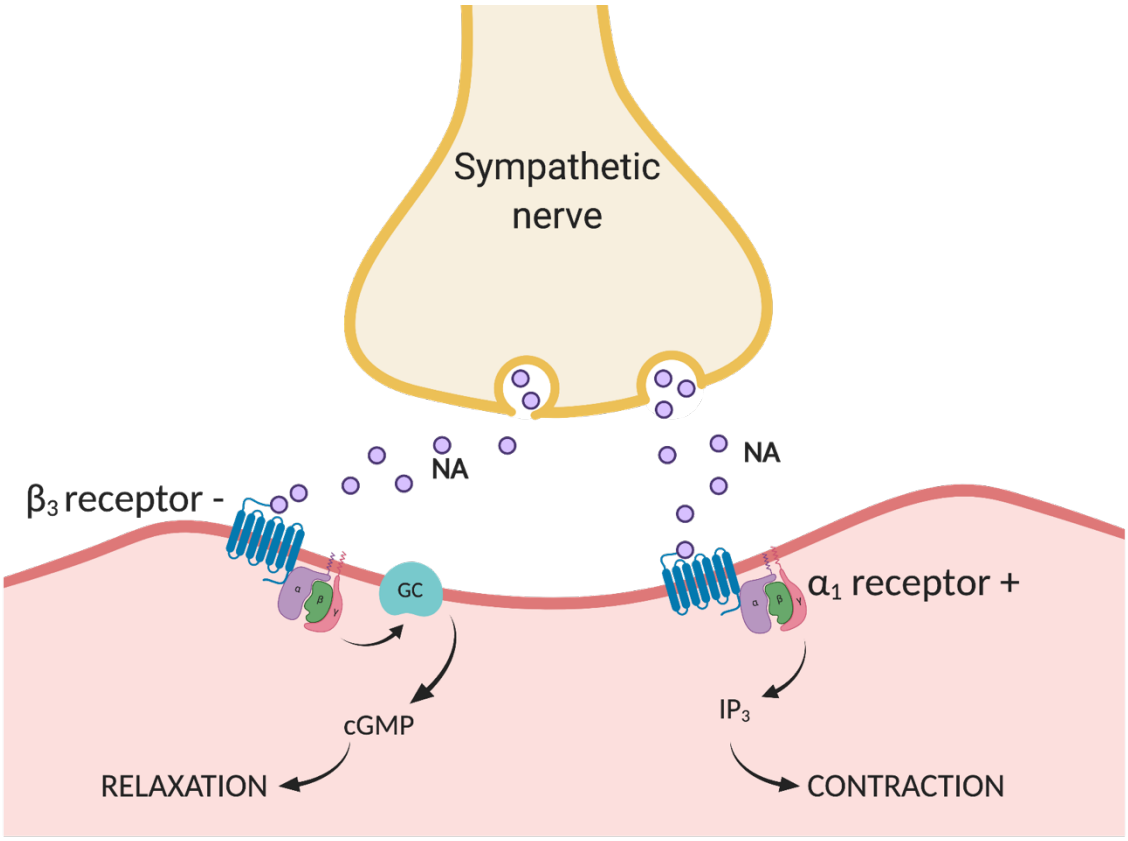

Figure 1.4: Inhibitory and stimulatory effects of noradrenaline on the detrusor and urethra. Sympathetic nerves (S), Noradrenaline (NA), Guanylate cyclase (GC), Cyclic Guanylate Monophosphate (cGMP), Inositol triphosphate (IP_3). (Created with BioRender.com by the author)

Parasympathetic Innervation

The parasympathetic innervation arises from the S2-S4 segments of the spinal cord and travels in the pelvic nerves and along to the pelvic plexus of the bladder wall (Fowler et al., 2008). The parasympathetic post ganglionic nerves release both acetylcholine (ACh) and non-adrenergic, non-cholinergic (NANC) neurotransmitters. The cholinergic transmission of ACh is the main excitatory mechanism of the human bladder, and acts at muscarinic receptors in the detrusor muscle (Andersson & Arner, 2004).

ACh activates predominately M_3 muscarinic receptors to initiate bladder contraction (Matsui et al., 2002). The role of M_2 receptors is not clear although there is evidence that there is an indirect 're-contraction' of the bladder via M_2 Muscarinic receptors. This

occurs by the reduction in adenylate cyclase activity which reverses the relaxation induced by β_3-adrenoreceptor stimulation (Chess-Williams, 2002). Activated muscarinic receptors primarily couple to the G_q-protein which activate phospholipase C (PLC) which produces IP_3 and diacylglycerol (DAG) from the membrane phospholipids phosphatidylinositol 4,5-biphosphate (PIP_2). This results in calcium (Ca^{2+}) release from the sarcoplasmic reticulum (SR), so that it may become available to calmodulin (CaM). When these molecules bind, myosin light chain kinase (MLC) is activated and phosphorylation activates myosin to bind to actin and cause contraction (Andersson & Arner, 2004) **(Figure 1.5)**.

Solifenacin, discussed in further detail later in this thesis, is an antagonistic drug that acts by binding M_3 muscarinic receptors and blocking the action of ACh. This results in decreased contractility of the bladder smooth muscle, thereby increasing urine storage.

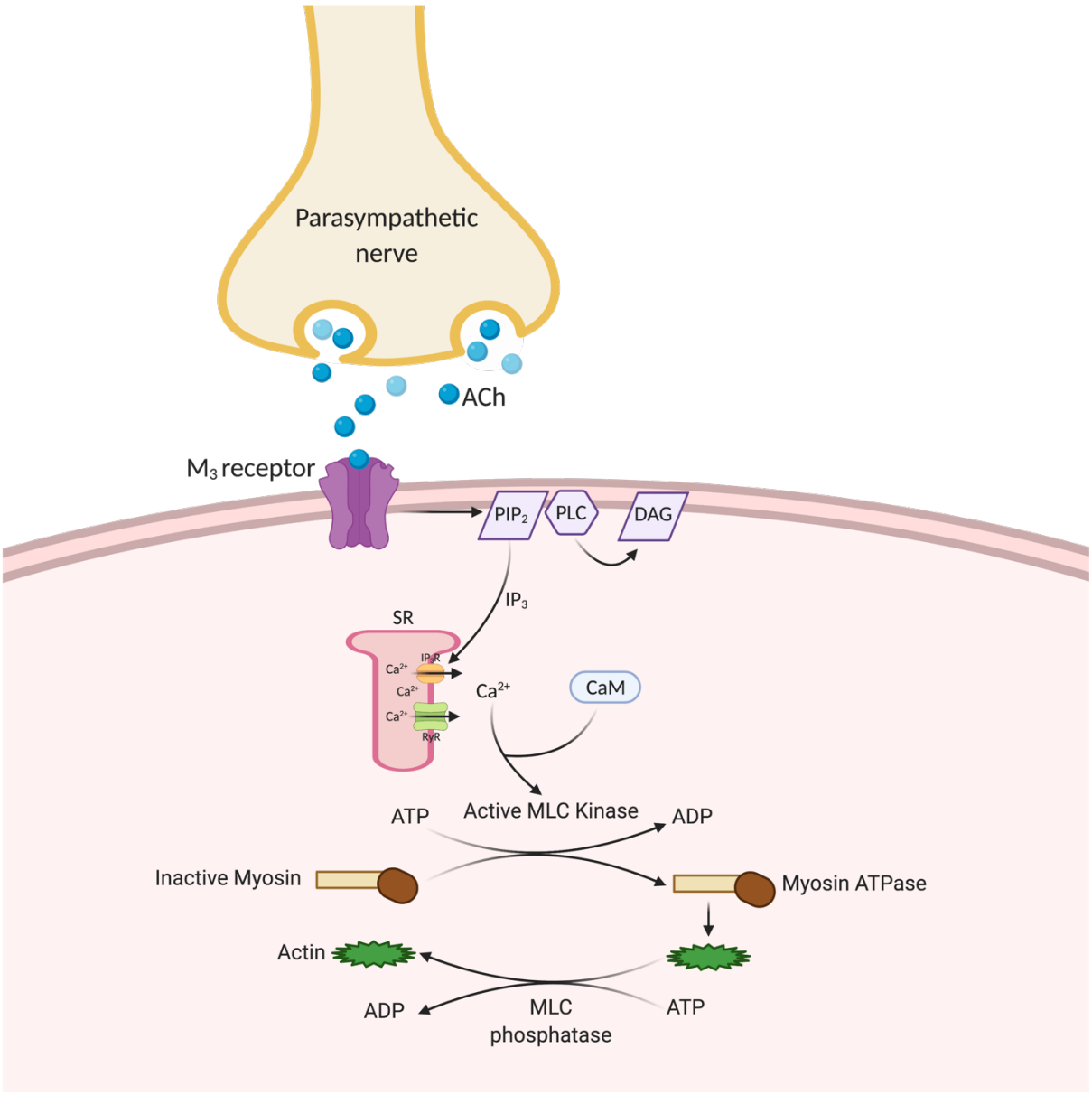

Figure 1.5: Diagram illustrating the contractile pathway of parasympathetic ACh neurotransmitter via muscarinic receptors of the detrusor smooth muscle. Acetylcholine (ACh), Adenosine triphosphate (ATP), Adenosine diphosphate (ADP), Calmodulin (CaM), Calcium (Ca^{2+}), Diacylglycerol (DAG), Inositol trisphosphate (IP_3), Muscarinic receptor 3 (M_3), Myosin light chain (MLC), Phosphatidylinositol biphosphate (PIP_2), Phospholipase C (PLC), Sarcoplasmic reticulum (SR). (Created with BioRender.com by the author).

Adenosine-tri-phosphate (ATP) is stored and co-released in conjunction with ACh to cause contraction of the urinary bladder (Vial, 2000). ATP is released from parasympathetic postganglionic neurons and can bind to either P2X or P2Y receptors, where P2Y receptors are metabotropic G-protein coupled and P2X are ligand-gated non-

specific cation channels. ATP activates the channels which causes and influx of sodium (Na^{2+}) and Ca^{2+}, depolarising the cell and activating the L-type Ca^{2+}channels. This generates and action potential for further influx of Ca^{2+} **(Figure 1.6)** (Fry, Meng, & Young, 2010).

Non-cholinergic bladder contractions have been reported to be mediated by ATP release onto the P2X purinergic receptors (Burnstock, 2001). Several studies of the rat and mouse urinary bladder have found that the P2X purinergic receptor mediated response may be responsible for up to 50% of the neurogenic bladder contraction (Hegde et al., 1998). In normal human bladder, P2X receptors are expressed but ATP does not appear to contribute to neurogenic contractions (Vial, 2000). In diseased states, however, the contribution of P2X receptor stimulation in contraction is significantly increased.

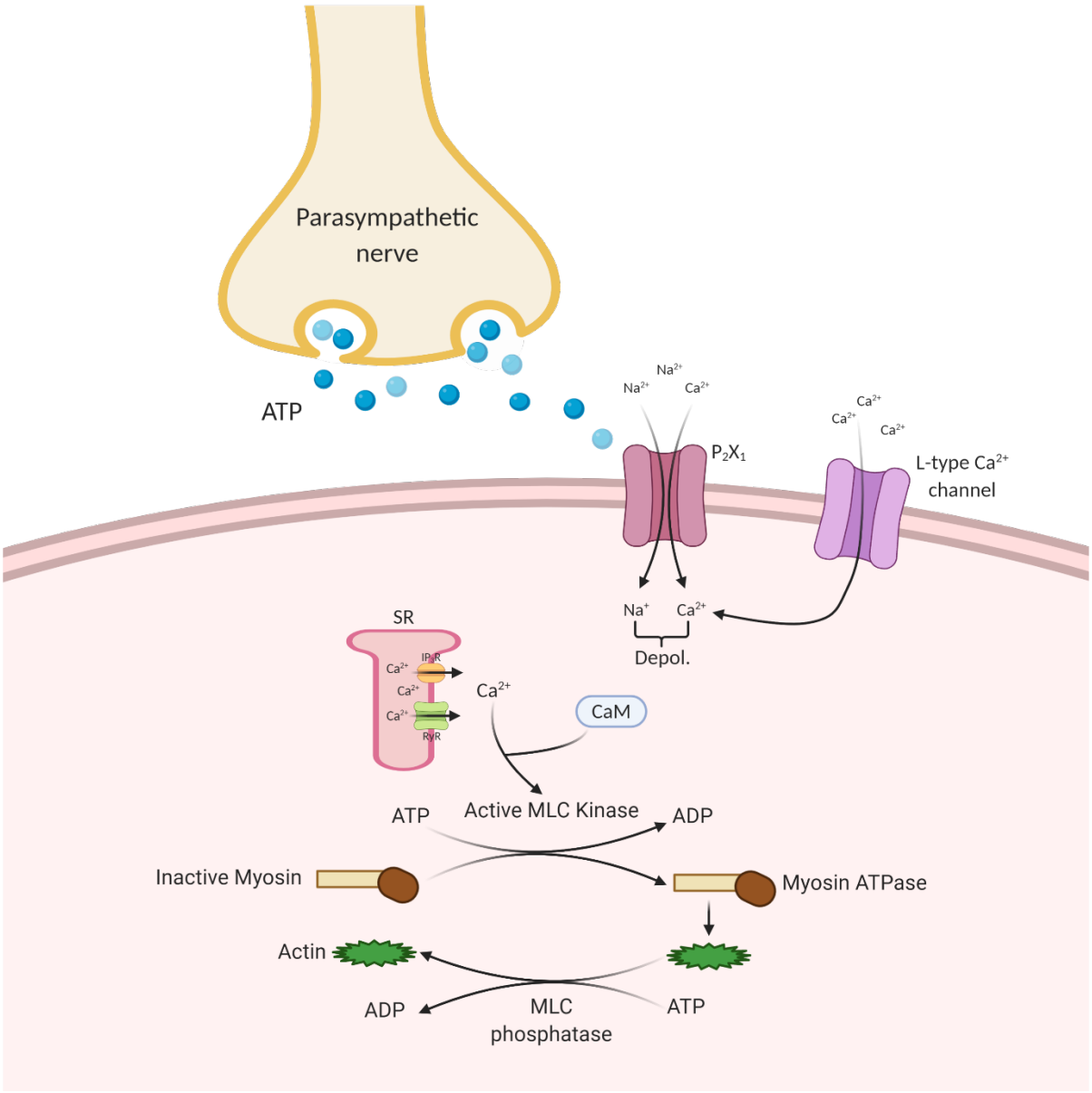

Figure 1.6: Diagram illustrating the contractile pathway of parasympathetic neurotransmitters ATP via purinergic receptors of detrusor smooth muscle. Adenosine triphosphate (ATP), Adenosine diphosphate (ADP), Calmodulin (CaM), Calcium (Ca^{2+}), Long lasting type Calcium channel (L-type Ca^{2+} channel), Myosin light chain (MLC), P2X Purinoceptor 1 (P_2X_1), Sodium (Na^+), Sarcoplasmic reticulum (SR). (Created with BioRender.com by the author).

Parasympathetic nerves also release nitric oxide (NO) which is responsible for the relaxation of the bladder smooth muscle during micturition (Sugaya, Nishijima, Miyazato, & Ogawa, 2005). The role of NO is still unclear, however, NO has also been shown to play

a direct role in the micturition reflex, by activating the cyclic GMP (cGMP) pathway in detrusor of rats (Caremel, Oger-Roussel, Behr-Roussel, Grise, & Giuliano, 2010).

The Urothelium

The bladder urothelium was once thought to act solely as a distensible barrier to the noxious effects of urine. It has since been established as a complex anatomical layer with a number of dynamic abilities, with one of the main abilities being its ability to act as a sensor to the intravesical environment (Winder, Tobin, Zupancic, & Romih, 2014) and the release of a number of chemical mediators that act on underlying cells as well as back on the urothelium itself **(Figure 1.7)**.

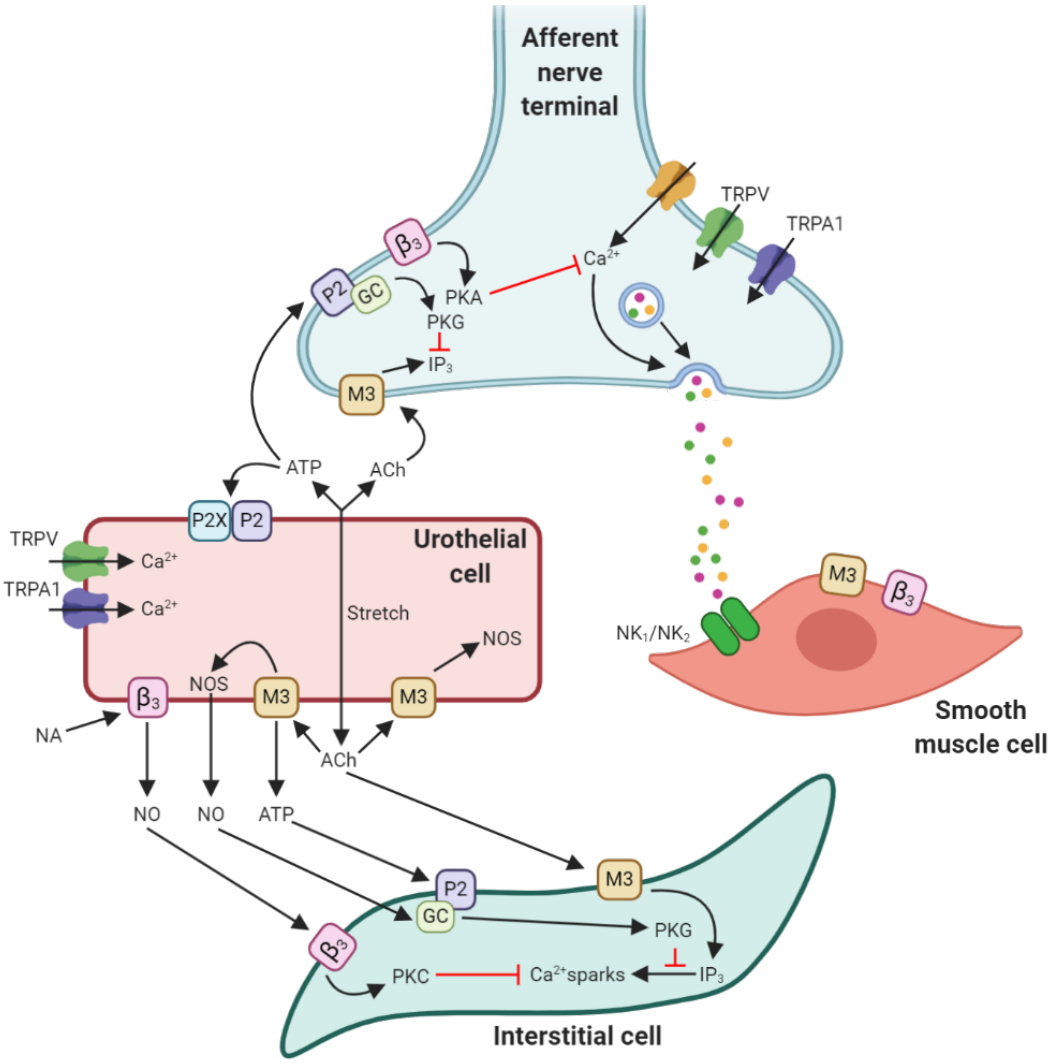

Figure 1.7: Illustration of proposed interactions between urothelial cells, bladder nerves, interstitial cells and smooth muscle. Acetylcholine (ACh), Adenosine triphosphate (ATP), β_3 adrenoceptor (β_3), Calcium (Ca^{2+}), G coupled protein receptor (GC), Muscarinic receptor 3 (M_3), Noradrenaline (NA), Neurokinin (NK1/NK2), Nitric oxide (NO), Nitric oxide synthase (NOS), Protein kinase C (PKC), Protein kinase G (PKG), Purinergic receptors (P2X/P2Y, P2), Transient receptor potential cation channel, subfamily A, member 1 (TRPA1), Transient receptor potential cation channel, vanilloid (TRPV). (Created with BioRender.com by the author).

Receptors and ion channels of the urothelium have been implicated in the mechanoceptive or nociceptive sensations. The urothelium contains a number of receptors including $P2X_{1\text{-}7}$, $P2Y_1$, $P2Y_2$ and $P2Y_4$ as well aa adrenergic α and β receptors, along with muscarinic receptors. Besides these main receptors, a group of receptors also

exist within the urothelium which are important for mechanoceptive and nociceptive responses. These receptors all belong to the transient receptor potential (TRP) channel family. TRPA1 is a receptor which is activated by noxious cold and stretch or swelling. The main function of this receptor is detrusor muscle contraction, urethral mechanosensation and bladder hyperreflexia **(Figure 1.7)**. There are 3 main TRPV receptors, which all respond to moderate to noxious heat. TRPV1 is involved in sensing bladder distention, bladder hyperreflexia and pain, and TRPV2 is responsible for urothelial cell mechanosensation, chemosensation and thermosensation. The final TRPV receptor, TRPV4, plays a role in detrusor contractility, bladder distension, voiding dysfunction, detrusor sphincter dyssynergia and pain (Merrill, Gonzalez, Girard, & Vizzard, 2016).

The urothelium also releases a range of chemical mediators and neurotransmitters. Stretch has been found to induce the release of ACh from the urothelium (Moro, Uchiyama, & Chess-Williams, 2011; Yoshida et al., 2006). ACh can be released in both a neuronal and non-neuronal manner. Neuronal release of ACh begins in the mitochondria when acetyl CoA is combined with choline to produce ACh. ACh is then transported in the synaptic vesicle to the synaptic cleft where, once released, it will bind to muscarinic receptors or be broken down by acetylcholinesterase. Choline is then transported back into the axon terminal by cotransport with sodium. The choline is then recycled and used to make more ACh (Nausch, Heppner, & Nelson, 2010). The mechanisms of non-neuronal ACh release, such as observed in the urothelium, continues to be investigated however, a study by Hanna-Mitchell et al. (2007) used cultured rat urothelial bladder cells and found high affinity choline transporters (CHT1), which are involved in synthesis of acetylcholine in the bladder. The study also found that the ACh synthesising enzymes such as choline acetyltransferase (ChAT) and carnitine acetyltransferase (CarAT) are

located in the urothelium. The same study also found that ACh release from the urothelium does not depend on vesicular storage of acetylcholine (Hanna-Mitchell et al., 2007). There is a suggestion that the synthesis of acetylcholine involves several steps beginning with the uptake of choline from the extracellular compartment via CHT1 and then formation of ACh with the cytoplasmic enzymes mentioned above, ChAT and CarAT. It is therefore unlikely that ACh is stored within vesicles in urothelial cells as several studies did not find the vesicular acetylcholine transporter (VAChT) present in the urothelial cells (Hanna-Mitchell et al., 2007; Lips et al., 2005; Wessler et al., 2001). Urothelial released ACh (non-neuronal) may target several different areas of the bladder, including nicotinic and muscarinic receptors on nerve terminals, detrusor muscle cells and urothelial cells (Birder & Andersson, 2013). ACh may have indirect effects by inducing the release of other substances such as NO, ATP and prostanoids (Nile, 2012). The urothelial release of ACh has been reported to increase with age in the human bladder (Yoshida et al., 2006) and may vary in diseased states. It is for this reason that it is believed that urothelial ACh may facilitate or inhibit afferent signalling depending on the pathology of the bladder (Winder et al., 2014).

Urothelial ATP is the most well studied mediator, although it is still not completely understood (Winder et al., 2014). Filling of the urinary bladder induces mechanotransduction pathways which are thought to be initiated by increased tension in the umbrella cells. These mechanical stimuli are likely to induce the stretch-activated release of ATP from the bladder urothelium (Cheng et al., 2011). Once outside the cell, ATP functions as a signalling molecule, modulating a range of cell functions via purinergic receptors (Burnstock, 2007). In the urothelium, ATP is released from the apical and basolateral surfaces and acts via $P2X_2$ and $P2X_3$ receptors on urothelial cells, interstitial cells and G-coupled protein receptors of the afferent nerve terminal (Wang et al., 2005).

ATP release has also been shown to be induced by other mediators that may be present in the urine, and by the release of mediators through nerve stimulation and other mediator release from the urothelial membrane (Sui et al., 2014).

Urinary ATP levels have been noted to increase in patients with bladder pathologies such as overactive bladder and interstitial cystitis. Reflex bladder activity has been shown to be modulated by an alteration of basal ATP release, thus suggesting that ATP may also play a role in control of micturition. ATP has recently been shown to be released from both vesicular and non-vesicular (pannexin channel) mechanisms (Beckel et al., 2015). What is unclear, however, is if these two pathways mediate the same downstream mechanisms. Physical stimuli, such as stretch, activates ATP release through pannexin channels while stimulation of Toll-like receptors (TLRs) activate vesicular release. When this is considered, ATP release can mediate separate downstream effects on bladder function depending on its mechanism of release (Beckel et al., 2018).

NO is formed from the amino acid L-arginine by the enzyme to nitric oxide synthase (NOS). Studies in cats, rats and rabbits have shown that urothelial cells release NO when stimulated by various chemicals and neurotransmitters such as ACh, capsaicin, nicotine and noradrenaline (Birder & Andersson, 2013). NO is thought to inhibit afferent nerve signalling. This was shown in a study using oxyhaemoglobin, a nitric oxide scavenger, where the absence of NO caused bladder overactivity, possibly via modulation of threshold firing (Yoshimura, Seki, & de Groat, 2001). Yoshimura et al. (2001), concluded that the cGMP pathway modulated the high voltage Ca^{2+} channels thereby affecting the firing threshold. NO has also been found to play different roles within the bladder depending on how it is synthesised. NO formed by inducible nitric oxide synthase (iNOS), may sensitize the bladder afferent nerves and thereby increase bladder contractility,

while NO formed by endothelial nitric oxide synthase (eNOS) may inhibit bladder contraction (Lee, Chiang, Tain, Wu, & Chuang, 2012).

Urothelial Derived Inhibitory Factor (UDIF)

The urothelium has the ability to modulate contractile responses of the detrusor muscle. Hawthorn, Chapple, Cock, and Chess-Williams (2000) found that the urothelium releases a diffusible inhibitory factor that inhibits contraction of the pig bladder detrusor muscle when the urothelium was stimulated with carbachol. Contractions of urothelium-denuded tissue were inhibited when a second bladder strip with intact urothelium was added to the organ bath, confirming the diffusible nature. The identity of the urothelial derived inhibitory factor was not concluded, but it was neither NO, gamma-aminobutyric acid (GABA), a catecholamine nor adenosine nucleotide (Hawthorn et al., 2000).

There seems to be controversy around the existence of UDIF in different species. While it has been confirmed in the pig urinary bladder, some studies show that UDIF is not present in the rat bladder, while others conclude that it is present (Munoz, 2010). The role of UDIF in the human bladder has been under discussion. Studies have observed the effects of NO, cyclooxygenase and β-adrenoceptors products on the bladder and concluded that there must be some other factor that is inhibiting detrusor contraction. It has therefore been hypothesised that another factor, distinct from NO, cyclooxygenase products, and β-adrenoceptors is contributing to inhibition of detrusor contractions (Chaiyaprasithi, Mang, Kilbinger, & Hohenfellner, 2003). While there has not been extensive investigation of the effect of age on UDIF, a study of urethral tissues of young pigs and older sows found that the urothelium and lamina propria have an inhibitory effect on the contraction of the urethra, which was not affected by age (Folasire, Chess-Williams, & Sellers, 2017).

Detrusor Muscle

The detrusor muscle makes up the majority of the bladder wall and is composed of bundles of muscle cells surrounded by connective tissue (Andersson & Arner, 2004). The lengthening and shortening of the muscle dictates the strength of contraction during bladder filling and voiding (McLafferty, Johnstone, Hendry, & Farley, 2014). Contraction of the bladder mainly occurs by ACh release from parasympathetic nerves onto muscarinic receptors, thereby stimulating emptying of the bladder. The location of the muscarinic receptor subtypes differs depending on the mode of action. M_1 and M_4 receptors are located of the pre-junctional nerve terminals (Sellers & Chess-Williams, 2012). M_2 receptors and M_3 receptors are located directly on the smooth muscle cells and while M_2 receptors are the predominant subtype, M_3 receptors are the main receptors mediating contraction (Yamanishi, 2001). During bladder filling, NA is released from sympathetic nerves, stimulating detrusor muscle relaxation by acting on β_3 adrenoceptors (Yoshimura & Chancellor, 2003).

Spontaneous Activity

Spontaneous activity occurs in the normal bladder, however, the role of spontaneous activity in micturition has not been defined (Kushida & Fry, 2016). Spontaneous contractions have been observed in many different models including, rat, pig, rabbit and mouse, both in *in vivo* and *ex vivo* preparations (Ng, de Groat, & Wu, 2006). In mice, in particular, the detrusor smooth muscle cells generate spontaneous action potentials at a low frequency (Meng, Young, & Brading, 2008). It has been noted in some studies that spontaneous contractions may be responsible for increased pressure during bladder filling, in some bladder disorders (Szigeti, Somogyi, Csernoch, & Szell, 2005).

It is known that detrusor overactivity may contribute to incontinence and large amplitude spontaneous bladder contractions evoke afferent nerve firing which may be linked to detrusor overactivity (McCarthy et al., 2009). Spontaneous activity or phasic contractions have been widely researched for their ability to contribute to bladder dysfunction. Prostaglandins have been found to increase the frequency of spontaneous contractions and the same study found that blocking P2X receptors results in decreased frequency of spontaneous activity (Kobayter, Young, & Brain, 2012). Another study has found that potassium channels have a fundamental role in stabilising detrusor smooth muscle excitability and if these channels are blocked, there is a reduction in spontaneous activity. Calcium transients were also observed to generate in detrusor muscle cells and propagated to neighbouring cells which suggests that spontaneous contractions pass through the bladder wall (Hayase, Hashitani, Kohri, & Suzuki, 2009).

PSYCHOLOGICAL STRESS

Psychological stress is known to affect a large portion of society. Stress cannot be defined by any one cause and depends greatly on a person's surroundings. Stressors, no matter what severity, have been documented to promote behavioural and psychological disturbances (Schneiderman et al., 2005).

Types of Psychological Stress

Stress can be broken down into two types: acute stress and chronic stress. Acute stress is often caused by reactive thinking to a situation and is usually brief while chronic stress occurs over a long period of time and is the most harmful form of stress. The longevity of the stress is often the cause of chronic depression and anxiety. Stressors such as anxiety and depression often develop after a negative life event. A long-term follow up study has observed that anxiety disorders often occur before depression (Breslau, Schultz, & Peterson, 1995). A number of studies have observed the difference between both stressors and concluded that activation of the stress response centres in the brain depends on the type of stress (Retana-Marquez et al., 2003). Therefore, chronic stress leads to an increase in activity of the hypothalamus-pituitary-adrenal (HPA) axis, leading to an increase in stress hormone release, discussed more below (Herman, 2013). There have also been studies which have observed that chronic stress leads to atrophy of the brain mass and a decrease in brain weight. These changes affect cognition and memory and often result in long-term effects on the nervous system (Reznikov et al., 2007).

Psychological Stress Pathways

Stress is generally defined as any form of stimulus that disrupts or affects the body's internal balance. The best studied component of the stress pathways of humans is the HPA axis. The HPA system is modulated through a negative feedback system to maintain stress hormone levels (Stephens & Wand, 2012). The paraventricular nucleus of the hypothalamus releases two hormones, corticotropin releasing factor (CRF) and arginine vasopressin (AVP). Both hormones stimulate the anterior pituitary to produce adrenocorticotropic hormone (ACTH). ACTH in turn, stimulates the adrenal glands, located atop of the kidneys, to synthesise glucocorticoids and catecholamines (Ranabir & Reetu, 2011).

Adrenaline is the primary catecholamine produced by the adrenal medulla. During stress, adrenaline is synthesised and transported into the blood stream and has key actions in a number of body systems including the cardiovascular system, increasing heart rate as well as the force of contraction. This increases cardiac output and increases blood pressure. In the respiratory system, adrenaline promotes bronchodilation, while in the digestive system, gastrointestinal (GI) function is inhibited (Goldstein, 2010) **(Figure 1.8)**.

Glucocorticoids are synthesised in the zona fasciculata of the adrenal gland from cholesterol. There are three glucocorticoids; cortisol, cortisone and corticosterone (Rosol, 2001). The main glucocorticoid varies between humans and mice, being cortisol and corticosterone respectively (Stephens & Wand, 2012) **(Figure 1.8)**. Cholesterol is the first step in the synthesis pathway to the dominant glucocorticoid (Ranabir & Reetu, 2011). In humans, cholesterol is converted to pregnenolone by cholesterol desmolase (Rosol, 2001). 17α-hydroxylase then converts pregnenolone to 17-hydroxypregnenolone, which is the first step of the synthesis pathway to occur in the

zona fasciculata. 3β-hydroxysteroid dehydrogenase then converts 17-hydroxypregnenolone to 17-hydroxyprogesterone. 21-hydroxylase converts 17-hydroxyprogesterone to 11-deoxycortisol and finally, 11β-hydroxylase converts 11-deoxycortisol to cortisol (Ranabir & Reetu, 2011). In the rodent, however, the pathway is slightly different. Cholesterol is converted to pregnenolone by cholesterol desmolase, and then is converted further to progesterone by 3β hydroxysteroid dehydrogenase. 21-hydroxylase converts progesterone to 11-deoxycorticosterone (Stephens & Wand, 2012). Finally, 11-deoxycorticosterone is converted to corticosterone by 11β-hydroxylase. Below is a summary of the cortisol and corticosterone pathway **(Figure 1.8)**.

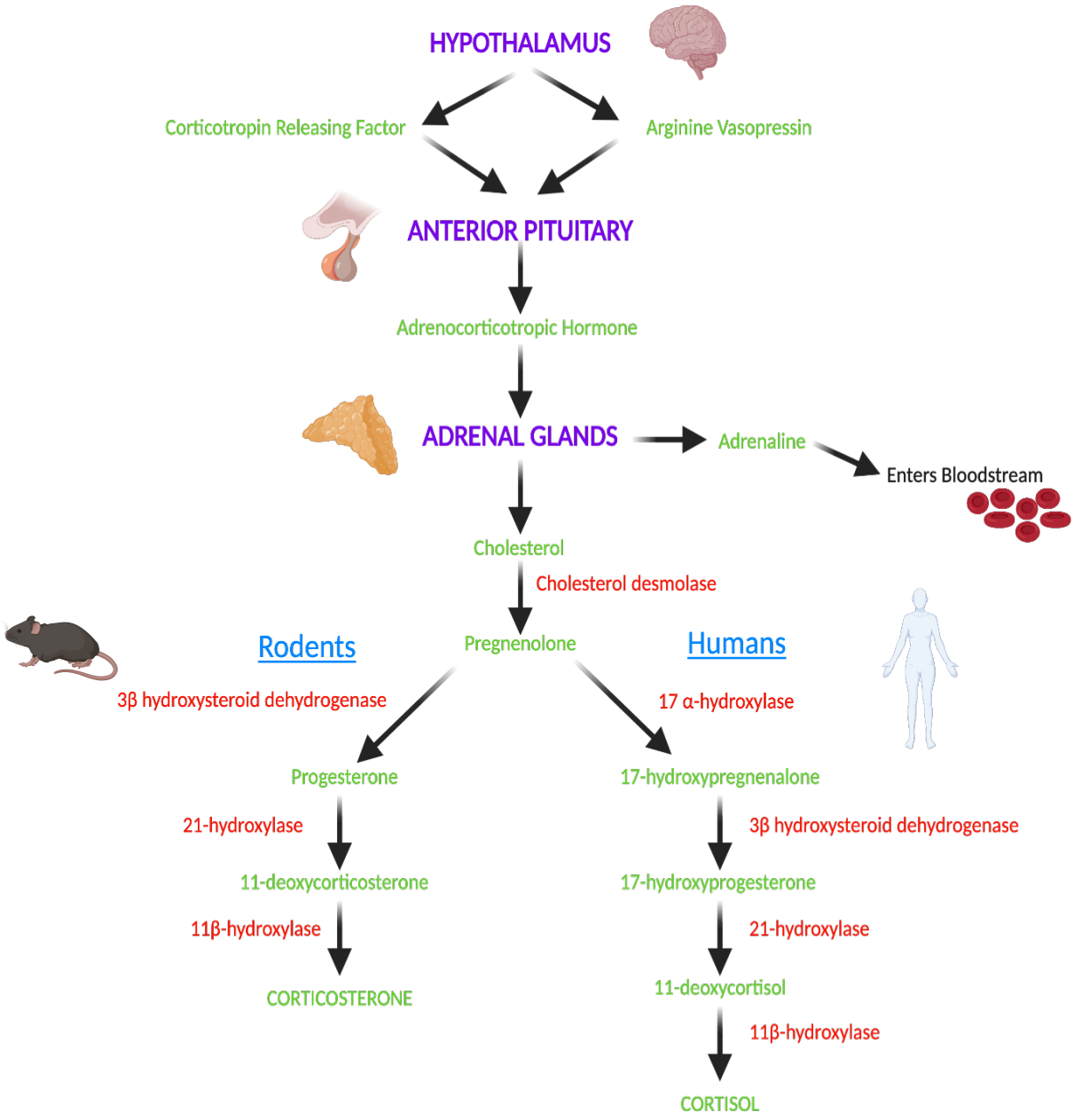

Figure 1.8: Psychological stress pathways of rodents and humans. (Created with BioRender.com by the author).

There are two types of receptors that cortisol binds to: mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs). Cortisol/corticosterone binds with higher affinity to MRs than GRs where MRs maintain low cortisol levels during normal daily circadian rhythm (Stephens & Wand, 2012). During a stressful situation, cortisol/corticosterone will activate GRs which results in the termination of the stress response. When stress hormones bind to GRs, there is a strong influence on

cardiovascular function, immunologic status, arousal, learning and memory (Ranabir & Reetu, 2011). Therefore, all of these responses are affected during stressful situations and too much or too little exposure to cortisol or corticosterone can have adverse consequences to general health and wellbeing (Stephens & Wand, 2012).

Psychological Stress and Disease

There have been many studies, looking at stress and disease outcomes. In many cases, psychological stress is a result of disease, however, there are cases where stress is known to cause disease (Cohen, Gianaros, & Manuck, 2016). Hormones have been shown to elicit effects on different parts of the central nervous system (CNS) and play a role in behaviour and cognitive function (de Kloet, 2000).

The relationship between stress and the immune system is one that has been discussed for years. Following stress, neuroendocrine and neural responses result in the release of corticotropin releasing hormone (CRH) and ACTH (Stephens & Wand, 2012). Early research has shown that the lymphatic system is also able to release these two mediators or other mediators that increase synthesis of these CRH and ACTH (Khansari, Murgo, & Faith, 1990). For example, thymus peptides, such as thymopoietin cause an increase in ACTH production (Goya, Castro, Hannah, Sosa, & Lowry, 1993). In severe cases, stress has been documented to lead to malignancy by suppression of the immune system. This occurs by stress decreasing the activity of cytotoxic T lymphocytes and natural killer cells thereby leading to proliferation of malignant cells (Reiche, Nunes, & Morimoto, 2004).

Several studies have observed the effects of stress on the cardiovascular system and have found these effects can be stimulatory or inhibitory in nature. Stress was observed to cause the autonomic nervous system to activate and indirectly affect the function of the cardiovascular system (Vrijkotte, van Doornen, & de Geus, 2000). If this occurs and

subsequently the sympathetic system is activated, there will be an increase in heart rate, strength of contraction, vasodilation in the arteries of skeletal muscles, contraction of arteries in the spleen and kidneys and decreased sodium excretion (Gordan, Gwathmey, & Xie, 2015). On the other hand, stress may cause activation of the parasympathetic system which leads to stimulation of the limbic system and reduced heart rate (Cohen et al., 2016).

Stress may also cause dysfunction in the gastrointestinal tract. Stress can affect appetite and is related to involvement of the ventral tegmental area (VTA) or the amygdala of the brainstem (Nasihatkon et al., 2014). Stress has been shown to affect the absorption process, mucus and stomach acid secretion, function of ion channels, intestinal permeability and GI inflammation (Qin, Cheng, Tang, & Bian, 2014). Crohn's disease and Inflammatory bowel disease are examples of diseases exacerbated by stress, where inflammatory markers are increased and there is recruitment of T lymphocytes. Lymphocyte aggregation leads to production of inflammatory markers which ultimately results in the appearance of inflammatory diseases (Konturek, Brzozowski, & Konturek, 2011). Stress is also able to affect the movement of the GI tract by slowing stomach emptying and accelerating colonic motility (Konturek et al., 2011). CRH has been shown, especially in the case of irritable bowel syndrome, to increase movement of the large intestine (O'Mahony et al., 2009).

The endocrine system and psychological stress have a complex relationship. In some cases, stress has many subtle effects on the activity of the endocrine system, while the endocrine system works to respond to stress (Ulrich-Lai & Herman, 2009). A small amount of stress stimuli has been shown to activate the HPA axis which from previous research has been shown to be intricately involved in the activation of different hormone secreting pathways (Stephens & Wand, 2012).

It was first reported by McEwen, Weiss, and Schwartz (1968) that the brain of rodents is capable of responding to glucocorticoids, thereby causing functional changes in the CNS. It was later determined that the hippocampus has both the mineralocorticoids, and glucocorticosteroid receptors, while other parts of the brain only have glucocorticoid receptors (de Kloet, Oitzl, & Joels, 1999). Stress has been shown to cause structural changes in different areas of the brain and chronic stress can lead to atrophy of the brain (Lupien, McEwen, Gunnar, & Heim, 2009). While it does depend on the type of stress, and length of time of the stressor, it is clear from many studies that stress can cause structural changes in the brain resulting in long-term effects on the entire nervous system (Reznikov et al., 2007). The structural changes of the brain include atrophy and neurogenesis disorders, and chronic stress has been shown to lead to an increase in plasma cortisol levels which has been shown previously to lead to a reduction in the number of dendritic branches, as well as changes in the synaptic terminal and decreased neurogenesis in the surrounding tissue (Sapolsky, Uno, Rebert, & Finch, 1990). Studies have reported that stress directly affects the functional aspects of the CNS, such as sensory, short-term and long-term memory (Wood, Dudchenko, Robitsek, & Eichenbaum, 2000). The conversion of short-term memory to long-term memory relies on the hippocampus which has been shown to have the highest density of glucocorticoid receptors (Asagloo, 2015). Noradrenaline is one of two factors involved in memory process and creates emotional aspects of memory in the basolateral amygdala area. The second factor of memory process is facilitated by corticosteroids. If the release of corticosteroids is earlier i.e., in a time of stress, it will cause inhibition of the amygdala and subsequent lack of memory processing (Joels, Fernandez, & Roozendaal, 2011). Animal models of psychological stress have been useful in determining the areas of the

brain affected by stress and the subsequent behavioural changes which contribute to the symptoms of depression and anxiety.

Brain Regions and Molecular changes Involved in Psychological Stress

There are several regions of the brain that are known to be affected by anxiety and depression. A number of clinical and experimental studies have observed changes both structurally and at the molecular level. The table below gives a brief overview of the type of animal stress models and the symptoms of depression observed as well as the effects on the brain and endocrine system. **Figure 1.9** also displays the brain regions highlighted in the table below.

Table 1.1: *Animal models of stress and the effects on the brain; Brain derived neurotropic factor (BDNF), Hypothalamic pituitary adrenal axis (HPA), Ventral tegmental area (VTA).*

Model	Symptoms	Affected areas/factors	References
Forced swim test	Behavioural changes	HPA axis, corticosterone, BDNF, zinc transporters	(Dou et al., 2014; Wang, Gu, et al., 2013)
Tail suspension test	Behavioural changes	HPA axis, corticosterone, BDNF	(Wang, Gu, et al., 2013)
Social defeat stress	Social avoidance	BDNF, VTA, astrocyte degradation,	(Krishnan et al., 2007; Vialou et al., 2014; Wang, Fanous, et al., 2013)
Reward based tests	Social avoidance, decreased sugar preference	VTA dopamine neurons	(Chaudhury et al., 2013)
Early maternal separation	Memory impairment, behavioural changes	Hippocampus, HPA axis, ATPase and Na^+, serotonin	(Sung et al., 2010; von Poser Toigo et al., 2012)

Olfactory bulbectomy	Anosmia, behavioural changes, irritability	HPA axis, hippocampus, amygdala, pre-frontal cortex, serotonergic system	(Song & Leonard, 2005; Yang et al., 2014)
Chronic unpredictable stress	Decreased sugar preference, locomotor activity and escape tendency	BDNF, hippocampus, pre-frontal cortex	(Banerjee, Hazra, Ghosh, & Mondal, 2014; Faria et al., 2014)
Learned helplessness test	Behavioural desperation	Serotonergic neurons, serotonin, CRH	(Maier & Watkins, 2005)

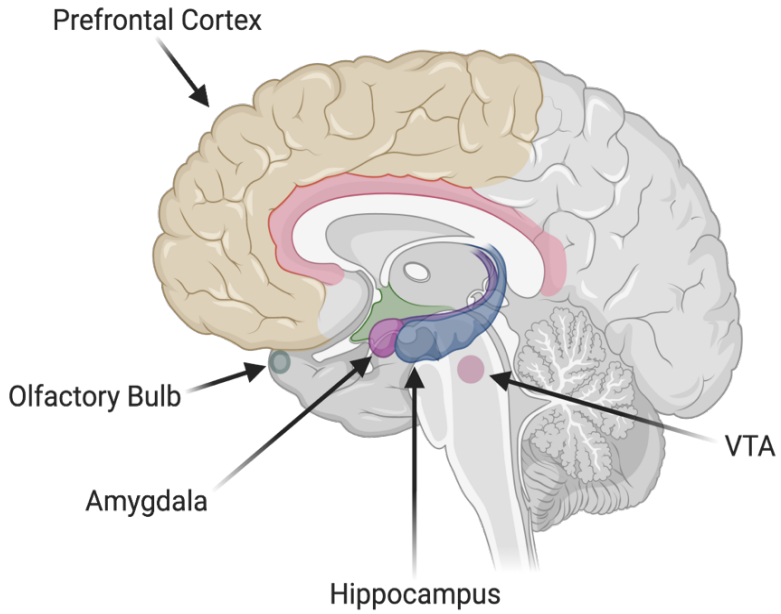

Figure 1.9: Diagram of the cortical and limbic systems involved in the stress response. Ventral Tegmental Area (VTA). (Created with BioRender.com by the author).

Changes in the HPA axis with stress has been linked to depression and anxiety in a number of studies. As stated previously, when the HPA axis is stimulated, corticosterone release is upregulated (Keeney et al., 2006). As seen above the forced swim test and tail suspension test upregulated corticosterone production, pointing to a link with the HPA axis (Wang, Gu, et al., 2013). The study also found a decrease of BDNF, which is a nerve

growth factor found in the brain and periphery, in both stressed groups. BDNF binds with its receptor, tropomyosin-related kinase B and activates a number of downstream signalling pathway (Duman & Voleti, 2012). While reduced BDNF in the hippocampus and prefrontal cortex, appears to play an important role in the cause of depression, a study by Duman and Voleti (2012), observed that genetic deletion of BDNF in rodents was insufficient to cause depressive symptoms and therefore would not be a target for anti-depressant treatment. A model of chronic unpredictable stress also found that BDNF protein and messenger RNA (mRNA) levels were decreased in the hippocampus and pre-frontal cortex (Banerjee et al., 2014). In contrast, sub-threshold social defeat stress has been observed to increase BDNF in the Ventral Tegmental Area of the brain stem (Krishnan et al., 2007). The study found an association between VTA-BDNF elevation after social defeat stress and increased vulnerability to psychostimulants (Wang, Fanous, et al., 2013). The VTA is also a brain region known to be affected by stress. Within this region of the brain there are dopamine neurons which play a crucial role in mediating the stress response. One study observed increased phasic firing of the VTA dopamine neurons in the reward-based tests of social defeat mice which induced a susceptible phenotype of depression with social avoidance and decreased sucrose preference observed (Chaudhury et al., 2013).

Another model of stress which has been used to measure depression is the early life stress model. This model involves mother rats being separated from their pups, one day after birth. Several studies have used this model to measure changes to the brain and one study found that repeated stress decreased cell proliferation and increased apoptosis in the hippocampus of the maternal rat brain (Sung et al., 2010). Another study of maternal separation observed a decrease in hippocampal sodium and adenosine triphosphatase (ATPase) activity and concluded that the withdrawal of rat pups causes mothers to be

more susceptible to neurochemical alterations (von Poser Toigo et al., 2012). Other studies have observed reduced hippocampal volume and atrophy of the hippocampal neurons (Balu & Lucki, 2009; Spalding et al., 2013). There is also evidence, from a rat model of depression, that zinc levels and zinc transporting-associated proteins are decreased in the hippocampus after psychological stress. As zinc is an important cofactor involved with biochemical processes in the brain, this change could greatly affect other molecular aspects (Dou et al., 2014).

Several rodent studies have observed synaptic loss in the cortical and limbic areas, especially in the prefrontal cortex and hippocampus regions that control emotion in response to psychological stress (McEwen & Morrison, 2013). Other brain imaging studies have shown that depression is associated with atrophy in prefrontal cortex and hippocampus volume (MacQueen & Frodl, 2011). One particular model of social-defeat stress has found that neuronal activity is reduced in the medial prefrontal cortex and astrocyte degradation is increased (Vialou et al., 2014). Other experimental studies of the prefrontal cortex investigated the role of astrocyte degradation in development of depressive symptoms and found a positive correlation between the factors (Domin, Szewczyk, Wozniak, Wawrzak-Wlecial, & Smialowska, 2014). The olfactory centre is a part of the limbic system, including the amygdala and hippocampus, which are known to contribute to emotional and memory components of behaviour (Song & Leonard, 2005). Bilateral olfactory bulbectomy is a procedure which results in the changes of behaviour often seen in patients with major depression. Studies using this method have found that neuronal degradation occurs throughout the pre-frontal cortex, hippocampus and amygdala resulting in dysfunction of the noradrenergic systems of the HPA axis (Song & Leonard, 2005; Yang et al., 2014).

Several models of stress have observed changes and deficiencies in serotonin within the brain. The study of early life stress found that serotonin depletion occurs over the course of the stress period (Sung et al., 2010). The model of stress involving olfactory bulbectomy has also demonstrated dysfunction of the serotonergic system within the HPA axis (Yang et al., 2014). The learned helplessness test had similar findings where the uncontrollable nature of the stressor lead to serotonergic neuron sensitisation and subsequent exaggerated release of serotonin (Maier & Watkins, 2005). These studies implicate serotonin as a major cause of behavioural changes observed in the rodents and indicate that the use of Selective serotonin reuptake inhibitors (SSRI's) may mediate these changes. SSRI's act by blocking reuptake of serotonin into the pre-synaptic neuron thereby increasing the amount of serotonin in the synapse. SSRI's will be further discussed in the following experimental chapters.

Psychological Stress and Bladder dysfunction

Bladder pathologies such as overactive bladder (OAB), interstitial cystitis and painful bladder syndrome (IC/PBS) are common in the general population and even more common with age (Teleman et al., 2004). OAB is characterised by urinary frequency, nocturia and urgency with or without incontinence. The cause of OAB has been a highly debated topic for many years, with two main theories being proposed; myogenic and neurogenic (Haferkamp, Dorsam, Resnick, Yalla, & Elbadawi, 2003), while in many instances the pathophysiology of OAB remains idiopathic. IC/PBS is a chronic condition which occurs when the lining of the bladder is constantly irritated (Torpy, 2012) and is characterised by pelvic pain and irritative voiding symptoms in the absence of lower urinary tract infection (Sant, 2002). The frequent urination and painful bladder that is

caused by interstitial cystitis results in a reduced quality of life (Siddiqui, Lagesen, Nederbragt, Jeansson, & Jakobsen, 2012).

It has been documented that psychological stress can impact several visceral functions with pathological consequences (Smith et al., 2011) and a body of clinical evidence exists linking bladder disorders with stress, anxiety and depression, including witness trauma (Lai, 2015). Stress appears to greatly influence the development of bladder symptoms, or worsen symptom severity (Minassian, Devore, Hagan, & Grodstein, 2013). Research also suggests that lower urinary tract dysfunction induces psychological stress symptoms in both children and adults (Sinclair, 2011; von Gontard, Baeyens, Van Hoecke, Warzak, & Bachmann, 2011). In spite of this, there is little research on the precise changes and underlying mechanisms of several bladder pathologies.

The two bladder pathologies mentioned above share some overlapping symptoms. The severity of these symptoms was assessed in a recent clinical study which compared stress levels of OAB, IC/PBS and control patients; using a series of questions all related to the patient's psychological stressors, and the number of times the patient needed to urinate. The study found that OAB patients reported psychological stress levels just as high as those reported by IC/PBS patients, with both groups of patients experiencing significantly higher stress levels compared to the healthy controls. This study reported that there is a positive correlation between perceived stress levels and urinary incontinence symptoms (Lai, 2015).

In another study by the same group, it was observed that half of OAB patients experienced anxiety like symptoms, while a quarter of the OAB patients experienced acute to moderate anxiety. OAB patients with anxiety reported having more severe OAB or incontinence symptoms which impacted their quality of life. The study concluded that there was a trend toward higher urinary frequency in patients with anxiety (Lai., 2016).

While the studies above have observed the correlation of stress and OAB at one time point, another study has observed incidence and remission rate of OAB over a 1-year period. Participants were all females who had returned from US Army deployment in the previous two years. The study looked at the impact of depression, anxiety, post-traumatic stress disorder (PTSD) and lifetime sexual assault on OAB incidence and remission rates. One thousand and seven women were eligible to participate in the study and of those women, 22% identified OAB symptoms at the beginning of the year. New OAB occurred more often in women with baseline anxiety at the beginning of the year. It was concluded that anxiety predicted 1-year incidence of OAB. It was also found that remission of OAB occurred less in those with baseline depression and anxiety (Bradley, 2017). The study demonstrates that anxiety can influence the natural progression of OAB.

Bladder dysfunction has been observed amongst various groups of people including sexual abuse survivors. Seventy-two percent of sexual abuse survivors report experiencing urinary incontinence symptoms compared to controls (22%) (Davila, Bernier, Franco, & Kopka, 2003). A number of clinical studies have observed the effects of stress on development of IC/PBS. One particular study investigated the correlation between childhood sexual trauma and IC/PBS incidence in later life. Female patients were questioned about their symptoms of IC/PBS and any childhood trauma. It was found that before 17 years of age, IC/PBS cases were increased in participants with a history of being raped or molested. Within this group, participants reported sensory pain, depression and poorer physical quality of life compared to the controls without IC/PBS symptoms. It was concluded that women with a history of sexual trauma are more likely to develop IC/PBS symptoms later in life (Nickel et al., 2011).

The clinical evidence reported above shows that there is a definite correlation between psychological stress and bladder dysfunction. While this gives a basic indication of the impact of stress on the bladder, the mechanisms underlying this dysfunction remains unknown. Some research groups have attempted to fill this knowledge gap using rodent models.

Experimental studies in rodents, see **Table 1.2**, typically focus on two different forms of psychological stress: water avoidance stress and social defeat stress. These models will be discussed in greater detail within the experimental chapters. Water avoidance stress replicates acute stress and is repeated for a length of time to simulate ongoing stress (Smith et al., 2011) while social defeat stress replicates social stress and as a model for depression (Hollis & Kabbaj, 2014). Most recent studies have focused on rats and have observed the effects of psychological stress on voiding function. This is where the knowledge gap becomes more apparent, where studies such as Wang et al. (2017) observe the effects of psychological stress on brain function related to regulation of micturition and Smith et al. (2011) observe changes in voiding activity. Isolated functional bladder responses have received little attention.

Wang et al. (2017) used female Wistar-Kyoto rats and exposed them to 10-days of water avoidance stress. Brain function was assessed using autoradiography and bladder function was assessed using cystometrograms and the study found that stressed animals demonstrated a decreased pressure threshold and visceromotor threshold triggering during the voiding phase, compared to the control rats. Water avoidance stressed rats also displayed greater activation of the cortical region of the central micturition circuit. Overall, the study found that water avoidance stress animals experienced visceral hypersensitivity during bladder filling. The stressed rats also had increased engagement of portions of the micturition circuit which lead to bladder contraction (Wang et al.,

2017). Smith et al. (2011) observed voiding frequency of stressed rats more closely than the previous study. In this study, it was found that rats exposed to water avoidance stress developed a significant increase in micturition frequency. The study also discovered that stress decreased latency to void, volume to void and the volume of the first void compared to the baseline of the control rats. Histology also revealed that in stressed rats, there was increased angiogenesis and increased activated mast cells (Smith et al., 2011). This finding was supported by Matos (2017), who also found that mast cell infiltration was increased in the mucosa of stressed rats. This study was based on the theory that chronic adrenergic stimulation produces pain and bladder changes mediated by alpha 1A adrenoceptor mechanisms. The study found that water avoidance stress reproduces signs of nociception and bladder changes that occur in patients with IC/PBS (Matos, 2017). The finding by Matos (2017) is supported by another study which observed chronically stressed Wistar Kyoto rats. The rats exposed to chronic stress exhibited enhanced responses to urinary bladder filling and significant stress induced changes in nociceptive responses to mechanical stimuli (Robbins, DeBerry, & Ness, 2007).

Other stress models have also been used to study the effects of stress models on bladder function. While this will be discussed in further detail in the following chapters, there are physiological bladder changes which occur with different forms of psychological stress. For example, the effect of social stress in particular has been found to decrease voiding frequency and increase voided volume while also increasing the amount of the stress neuropeptide CRF mRNA in the Barrington's nucleus neurons located within the pontine micturition centre (PMC) (Butler et al., 2018). This corresponds to the study by Mann (2015) which found that chronic social defeat results in an altered voiding phenotype indicative of urine retention. Studies have also found that this altered voiding phenotype corresponds to bladder wall remodelling, increasing bladder mass (Chang et al., 2009).

Contradictory to this, another study has found that social stress induced TRPV1-dependent nerve activity leading to increased urinary frequency and an overactive bladder phenotype (Mingin et al., 2015). The neonatal maternal stress model has also been investigated and was found to increase bladder hypersensitivity in female mice. Hypersensitivity was further exacerbated by water avoidance stress (Pierce et al., 2016). See **Table 1.2** below for abbreviated list of both clinical and experimental research on psychological stress and bladder dysfunction.

Both human and animal studies consistently establish the effects of psychological stress on bladder function, however, despite this growing clinical and experimental evidence, the precise changes and underlying bladder mechanisms have received very little attention. In addition, very few studies to date have assessed the potential benefits of current drug treatments in stress induced bladder dysfunction. One study looked at the effect of mirabegron, β_3 adrenoceptor agonist, solifenacin, M_3 muscarinic antagonist and duloxetine, serotonin and norepinephrine reuptake inhibitor (SNRI), on reducing overactive bladder correlated with psychological stress and found that the only drug that made a significant difference was duloxetine. In this case, Duloxetine was able to reverse the symptoms of overactive bladder co-existing with depression via an action in central nervous pathways (Wrobel et al., 2020). The effect of pharmaceutical drugs on bladder dysfunction caused by psychological stress will be further discussed in Chapter 5.

Hypothesis and Overall Aim

Psychological stress and bladder dysfunction affect a large proportion of society however, it is still unclear as to the exact effects of psychological stress on bladder function. It is hypothesised that due to the known influence psychological stress has on other disease and pathologies, it will also contribute to bladder dysfunction. If this is the case, it can then be postulated that both psychological and bladder pharmaceuticals may have an effect in reducing this dysfunction.

This thesis aims to further investigate the pathophysiological pathways and increase knowledge of the mechanisms causing changes in lower urinary tract (LUT) function following stress. It also aims to provide sufficient evidence as to the most appropriate and beneficial pharmacotherapies for use in patients with urological dysfunction associated with chronic psychological stress.

Table 1.2: **Comparison of clinical and experimental studies looking at the effects of psychological stress on bladder dysfunction.**			
Author, year, journal	Title	Experimental Model	General conclusions
Clinical Studies			
Bradley, et al. 2017 – American Journal of Obstetrics	Longitudinal associations between mental health conditions and overactive bladder in women veterans	Computer-assigned telephone interviews at enrolment and 1 year later of female veterans	- New OAB occurred in women with baseline anxiety - Anxiety and lifetime sexual assault predicted 1-year incident OAB - Anxiety, depression and prior sexual-assault influenced natural progression of OAB
Nickel, et al. 2011 – Canadian Urological Association	Childhood sexual trauma in women with interstitial cystitis/bladder pain syndrome: a case control study	Psychological phenotyping, psychosocial parameters and quality of life questionnaires of female IC/PBS patients	- IC/PBS cases reported higher prevalence of rape and molestation compared to controls - IC/PBS cases reported greater sensory pain, depression and poorer physical quality of life - Childhood trauma is more common in IC/PBS patients
Lai, et al. 2016 – Elsevier Inc.	The Relationship Between Anxiety and Overactive Bladder or Urinary Incontinence Symptoms in the Clinical Population	Anxiety and depression were assessed using Hospital Anxiety and Depression Scale in patients diagnosed with OAB	- Half of OAB patients had anxiety while a quarter had symptoms of anxiety - OAB patients have psychosocial difficulties

Lai, et al. 2015 – BMC Urology	Correlation between psychological stress levels and the severity of overactive bladder symptoms	Patients with OAB were assessed using perceived stress scale	- OAB patients reported psychological stress levels as high as IC/PBS patients - Positive correlation between perceived stress levels and urinary incontinence symptoms
Davila, et al. 2003 – The Journal of Urology	Bladder Dysfunction in Sexual Abuse Survivors	Sexual abuse survivors completed a questionnaire regarding OAB symptoms and sexual abuse history	- Sexual abuse survivors had a significantly higher rate of OAB symptoms
Gontard, 2012 – Neurourology and Urodynamics	Does psychological stress affect LUT function in children?	Psychological evaluation using parental behavioural questionnaires	- Psychological symptoms may be a result of lower urinary tract dysfunction in children
Sinclair, et al. 2011 – The Obstetrician and Gynaecologist	The psychosocial impact of urinary incontinence in women	Review of results from psychosocial questionnaires	- Incontinent women often have anxiety and embarrassment with their condition - Women's sexual function and relationships are often affected
Experimental Studies			
Wang, et al. 2017 – Public Library of Science	Effects of water avoidance stress on peripheral and central responses during bladder filling in the rat.	Water avoidance stress - Female Wistar-Kyoto rats	- Decreased pressure threshold for triggering the voiding phase - Greater activation of the cortical regions of micturition circuit

Matos, et al. 2017 – Naunyn Schmiedebergs Archives of Pharmacology	The water avoidance stress induces bladder pain due to a prolonged alpha1A adrenoceptor stimulation.	Water avoidance stress - Female Wistar rats	- Increased urinary noradrenaline - Decreased mechanical pain threshold -Induced lymphocytic and mast cell infiltration in mucosa - Alpha 1A adrenoceptor stimulation plays an important role in bladder pain in rats
Yamamoto, et al. 2012 – International Journal of Urology	Water avoidance stress induces frequency through cyclooxygenase-2 expression: a bladder rat model.	Water avoidance stress - Rat	- Shorter voiding intervals and less volume in Water Avoidance Stress (WAS) mice - Higher levels of cyclo-oxygenase 2 (COX-2) protein in WAS bladders
Smith, et al. 2011 – Journal of Urology	The effects of acute and chronic psychological stress on bladder function in a rodent model	Water avoidance stress - Wistar rats	- Micturition frequency, interval and volume
Lee, et al. 2015 – Physiology Behaviour	Chronic psychological stress in high-anxiety rats induces sustained bladder hyperalgesia	Water avoidance stress - Female Wistar-Kyoto rats	- Increased pain response - Decreased response to mechanical hind paw stimulation - Increased visceromotor responses to ice water testing
Wood, et al. 2009 – American Journal of Physiology	Social stress-induced bladder dysfunction: potential role of corticotropin-releasing factor	Social defeat stress - Male rates	- Urinary retention - Increased bladder size - Increased bladder capacity

Robbins, 2007 – Physiology and Behaviour	Chronic psychological stress enhances nociceptive processing in the urinary bladder in high-anxiety rats	Water avoidance stress – Female Wistar-Kyoto rats	- Stressed rats experienced increased responses during urinary bladder filling - Chronic stress enhanced bladder nociceptive responses
Mann, etal. 2015 – Physiology and Behaviour	Chronic social defeat, but not restraint stress, alters bladder function	Social defeat stress – C57BL/6 male mice	- Decreased voiding frequency - Bladder wall hypertrophy
Chang, etal. 2009 – American Journal of Physiology, Renal Physiology	Social stress in mice induces voiding dysfunction and bladder wall remodelling	Social defeat stress – FVB male mice	- Increased bladder mass - Decreased voiding and increased voiding at micturition
Butler, etal. 2018 – Physiology and Behaviour	Murine social stress results in long lasting voiding dysfunction	Social defeat stress – C57BL/6 male mice	- Increased CRF mRNA in Barrington's nucleus - Decreased voiding frequency accompanied with increased voided volume
Mingin, etal. 2015 – American Journal of Physiology	Social stress in mice induces urinary bladder overactivity and increases TRPV1 channel-dependent afferent nerve activity	Social stress – C57BL/6 male mice	- Increased urinary frequency - Increased TRPV1 dependent afferent nerve activity

CHAPTER 2: MATERIALS AND GENERAL METHODS

This chapter will specify the materials and general methodology used throughout the studies. Specific information regarding the experimental stress protocols will be detailed in the subsequent experimental chapters.

ANIMALS

Approval for this project was obtained from the University of Queensland Animal Research Ethics Committee (BOND/536/17). Adult female or male C57BL/6JArc and ARC(S) mice were obtained from the Animal Resource Centre (Canning Vale, WA, Australia). Mice were housed for one week prior to experimental use in a controlled environment with 12-hour light/dark cycles, temperature 23^{o}C with free access to food and water.

VOIDING PATTERN ANALYSIS

Voiding pattern analysis (VPA) is a semi-quantitative method that has been used previously to assess changes in voiding behaviour in rodents and the method used here has been previously used by our research group (West, Lang, Sellers, Chess-Williams, & McDermott, 2018). Voiding pattern analysis was generally carried out as stated here, apart from the recovery protocol which was undertaken for 20 days. This change is discussed in more detail in Chapter 4 part 2.

Voiding pattern analysis was performed prior to (baseline) and at intervals (1, 3, 5, 7 and 10-days) during and following the stress protocols. Standard mouse cages were lined with 'Filtech' hardened ashless filter paper, Quantitative 2 μm grade 225. The mice were placed individually into the lined cage for 4 hours at the beginning of the light cycle, 7am. During this time, the mice were supplied with food and drinking water. Water bottles were weighed before and after voiding pattern analysis to monitor water consumption.

After the 4-hour period, VPA filter papers were collected. Faecal pellets were also collected after the analysis period and were dried, counted and weighed. Voiding events were quantified by assessing urine spots, detected using a Molecular Imager ChemiDoc XRS ultraviolet transilluminator (#720BR1293 BioRad, California USA). The papers were photographed, digitized, and then analysed using Image J software, considering both the size (surface area) and number of voids. **Figure 2.1** shows two representative voiding pattern analysis photographs of **A**, control female mice and **B**, stressed female mice.

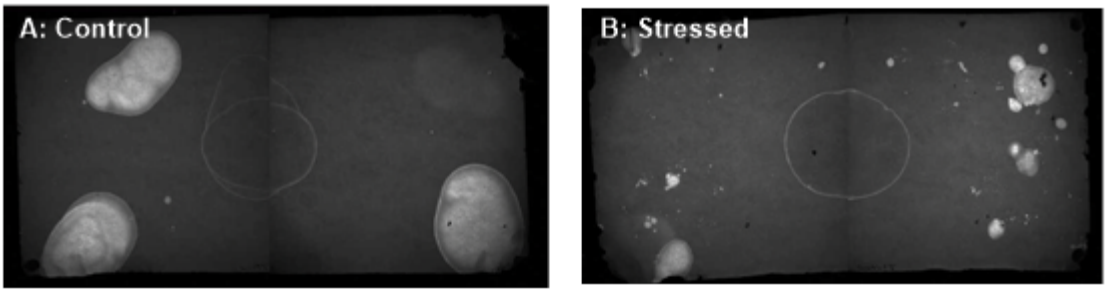

Figure 2.1: Representative voiding pattern analysis images from A) control and B) water avoidance stress (stressed) animals at day 5 (Photographs taken by the author).

BLOOD COLLECTION AND PLASMA CORTICOSTERONE MEASUREMENTS

At the end of the experimental protocol, mice were euthanized by cervical dislocation and blood was collected for quantification of plasma corticosterone. Five hundred μL of blood was taken from the thoracic cavity and placed into a heparin blood collection tube for centrifugation. The blood was centrifuged for 15 minutes at 900 rpm to separate the blood cells from the plasma. The plasma was then collected, placed into an Eppendorf and frozen at -80^{o}C.

A Corticosterone Competitive ELISA kit (Invitrogen) was used to quantify the amount of corticosterone in the plasma of stressed and control mice and was performed according to the manufacturer's instructions. To perform the assay, corticosterone stock solution

was used to make up 8 standards and 50µL of standards or samples were added to each well in duplicate. Seventy-five µL of ELISA assay buffer was added to one well to detect non-specific binding (NSB). Twenty-five µL of the ELISA corticosterone conjugate was then added to each well, followed by another 25 µL of ELISA corticosterone antibody to all wells, except the wells to detect NSB. The plate was then incubated for 1 hour at room temperature on a plate shaker. After incubation, the solution was aspirated and washed from the wells 4 times with 300 µL of wash buffer. Once washed thoroughly, 100 µL of tetramethylbenzidine (TMB) substrate was added to each well and incubated for a further 30 minutes without shaking. After the second incubation, 50 µL of ELISA Stop Solution was added to each well. The plate was gently mixed until the solution turned from blue to yellow (Chemical, 2018).

The absorbance was then read on the Modulus Microplate Multimode Reader (Turner Biosystem inc.) at 450 nm, within 10 minutes of applying the Stop Solution. Once read, NSB readings were subtracted from all samples and standards. A four-parameter curve fit was produced using Prism 8 software and concentrations of unknown samples were generated from the standard curve. An example of the standard curve is given below in **Figure 2.2**.

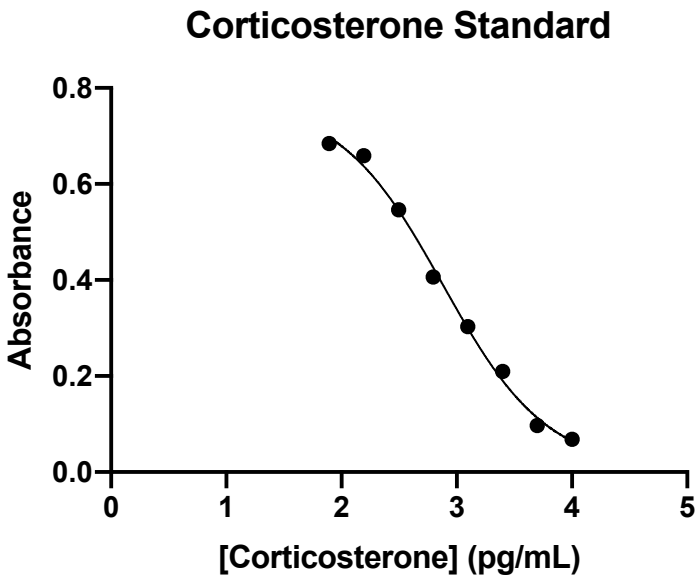

Figure 2.2: Representative Corticosterone standard curve prepared using Corticosterone Competitive ELISA kit, absorbance analysed using a Modulus Microplate Reader.

GENERAL WHOLE BLADDER PREPARATION

A whole bladder preparation was used for functional bladder studies. The protocol was previously used by our research group (West et al., 2018). Mice were sacrificed using cervical dislocation and placed onto a dissection board. Surgical grade scissors were used to cut through layers of fur and skin of the mouse below the rib cage. This process exposes the gastrointestinal tract which was then lifted out of the body and removed by cutting through colon and connective tissue. The abdominal region of the mouse was then separated from the upper body of the mouse by cutting through the spine, above the kidneys. The lower half was then turned to remove the skin and fur. The tail and lower limbs were then removed, and the abdominal section of the animal was placed into Krebs-bicarbonate solutions (8mL) and gassed with 95% O_2 and 5% CO_2.

Using a dissection microscope, surrounding connective tissue was removed to expose the ureters. Note that sex differences will be described in following chapters. The ureters were then ligated using sewing thread. The connective tissue above the pubic symphysis

was then cut away until the cartilage of the pubic symphysis was visible. Once located, the pubic symphysis was cut down the middle and removed. The external urethral orifice was removed and, using 2x magnification of the microscope, the three-way catheter was inserted through the urethra and up into the bladder. The urethra was then ligated by cutting up the vagina on either side of the urethra, being careful not to release the catheter. At this time, the ureters were then ligated a second time closer to the bladder. This was to ensure that the ureters were completely secured before the bladder was removed from the abdominal cavity. Once the bladder was removed as pictured in **Figure 2.3.B**, it was placed into an 8 mL bath of Krebs-bicarbonate solution at 37^0C.

The three-way catheter was attached to an infusion pump filled with Krebs-Bicarbonate solution and was set to deliver 30 µL per minute of Krebs to the bladder. Also attached was a pressure transducer which measured the pressure inside the bladder and transmitted this to the Lab Chart program for analysis. An outflow syringe was also attached to allow intraluminal fluid to be collected **(Figure 2.3A)**. The bladder was filled to 40 mmHg to assess viability and catheterisation.

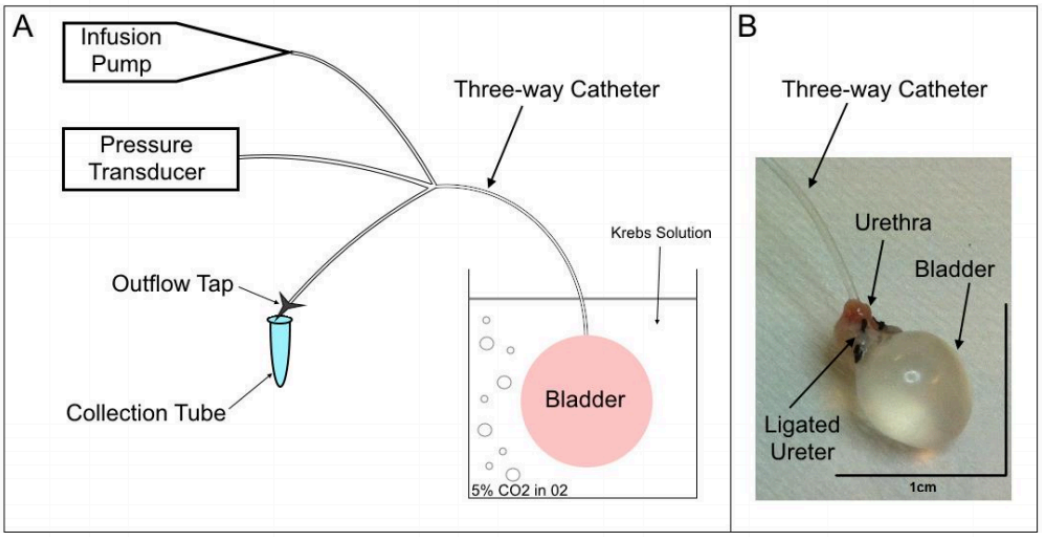

Figure 2.3: (A) Schematic displaying the three-way catheter set up. (B) Whole bladder preparation attached to three-way catheter. (Reproduced with permission from Farr (2015)).

FUNCTIONAL PHYSIOLOGICAL STUDIES

Collection of Intravesical and Serosal Fluid to Quantify Mediator Release

Once assessed as viable, the bladder was emptied for 10 minutes. The bladder was then filled to 20 mmHg twice, and at this time samples of intraluminal and serosal fluid were collected. The outflow tap allowed for intraluminal fluid to be collected during the emptying period after filling to 20 mmHg. 800 µL of serosal fluid was also collected from the 8 mL bath at a constant sample site, beside the bladder. Samples were stored at -80^{0}C for later assay of ATP and ACh. The bladder was filled once more to 20 mmHg and left to equilibrate for 30-minutes. The final fill to 20 mmHg was quantified and assessed as a pressure-volume relationship to measure bladder compliance. The 30-minute equilibration period was used to assess the stretch-relaxation of the bladders by measuring the time versus pressure relationship. The amplitude and frequency of spontaneous activity was also assessed during this time. An example of spontaneous activity is given below in **Figure 2.4**.

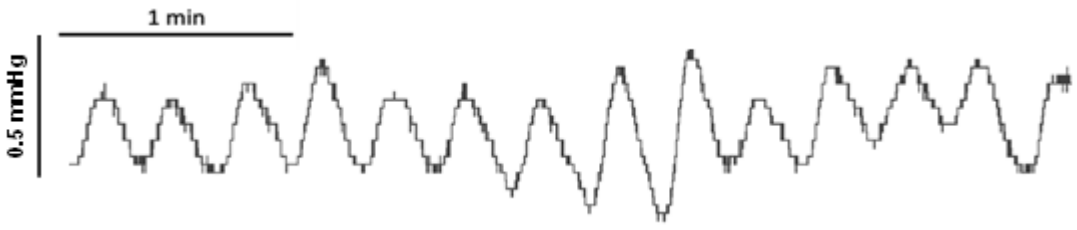

Figure 2.4: Representative trace showing amplitude and frequency of spontaneous activity in a control animal.

Electrical Field Stimulation

To investigate the effects of stress on nerve-mediated responses of the bladder, electrical field stimulation (EFS) was performed after the 30-minute equilibration period. Platinum electrodes attached to an electrical stimulator, were placed into the bath to sit

on either side of the bladder, allowing for an electrical field to be passed through the bladder. After washing with Krebs-bicarbonate solution, the bladder was electrically stimulated (50 V), every 100 seconds for 5 seconds, at 1, 5, 10, and 20 Hz. At each frequency, the bladder was stimulated at least 4 times, until 3 consistent responses were achieved. The contractions were measured as pressure change from baseline and an example of frequency-response is given below in **Figure 2.5**.

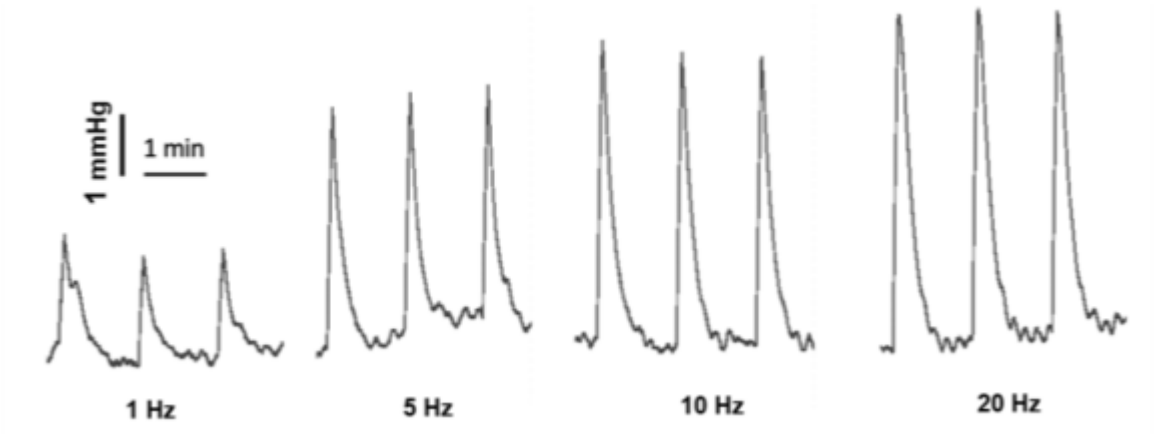

Figure 2.5: Representative frequency response trace in an isolated bladder from a control animal during electrical field stimulation for 5 seconds, every 100 seconds. Responses to 1, 5, 10 and 20 Hz are shown, each stimulus being 0.1 ms duration at 50 V: Hertz (Hz).

EFS was also completed at 20 Hz in the absence and presence of pharmacological agents towards the end of the experiment. The first agent that was added to the bath was the competitive inhibitor of nitric oxide synthase, N_{ω}-nitro-L-arginine (L-NNA) (100 μM). After 4 contractions at 20 Hz, the muscarinic receptor antagonist atropine (1 μM) was added to the bath and the bladder was again stimulated at 20 Hz. The EFS stimulator was switched off and the purinergic agonist alpha, beta-methylene adenosine triphosphate (αβ-mATP) (1 mM) was added to the bath twice and left for 5 minutes to desensitize the purinergic receptors. EFS was then repeated in the presence of αβmATP and after 4 contractions, tetrodotoxin (TTX; 1 μM) was added to the bath while EFS continued. It is important to note that throughout the EFS process with pharmacological agents, the

bladder was not washed. The bladder was only washed after EFS stimulation with TTX. After this, a 60 mM potassium chloride (KCl) solution was used to fill the entire bath, to measure a final contraction.

Response to Pharmacological Agents

To investigate the effects of stress on receptor-mediated bladder responses, pharmacological agents were added to the bath in order to act on the muscle and subsequent pressure changes were measured. Contractile responses to purinergic stimulation were assessed using ATP (10 mM) and αβ-mATP (1 mM). After stimulation with ATP the bladder was washed and the muscarinic agonist carbachol was added to the bath in 3-fold incremental concentrations to produce a cumulative concentration-response curve **(Figure 2.6)**. The maximum carbachol dose concentration examined was 6.84 μM because concentrations above this were found in preliminary studies to desensitize tissues. Once responses were recorded, the bladder was washed with fresh Krebs-bicarbonate solution and until pressure returned to initial baseline.

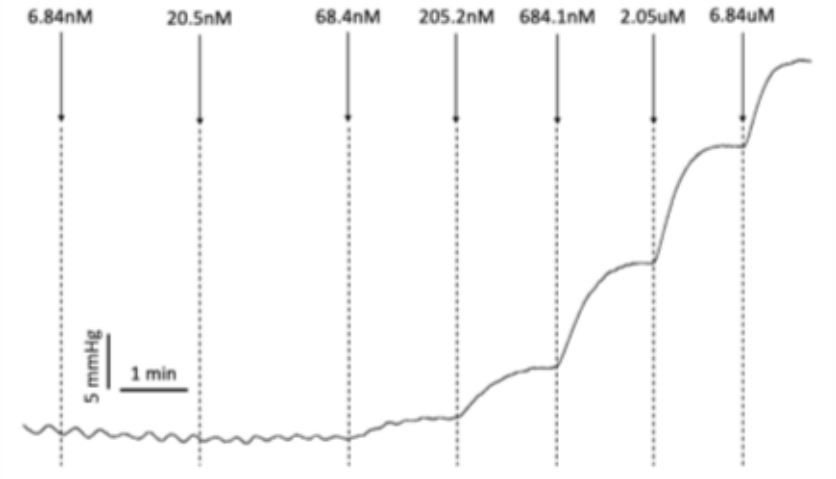

Figure 2.6: Representative trace showing intravesical pressure responses of a control isolated whole bladder preparation to the muscarinic agonist carbachol.

Once stable, 684.1 nM of carbachol was added to increase initial pressure. The bladder was left for 15 minutes to stabilize before obtaining an isoprenaline concentration-response curve. During this 15-minute period, the frequency and amplitude of carbachol induced phasic contractions were measured. Once stable isoprenaline, a non-selective β-adrenoreceptor agonist which causes bladder relaxation, was added to the bath in 3-fold incremental concentrations. Once the isoprenaline curve was recorded, the bladder was washed again with fresh Krebs-bicarbonate solution and allowed to return to baseline pressure. All contractions and relaxation responses were measured as a change in pressure from baseline.

UROTHELIAL MEDIATOR ASSAYS

Quantification of Acetylcholine

To measure ACh present in the intraluminal and serosal samples, the Amplex Red Acetylcholine (Molecular Probes) Assay kit was used according to the manufacturer's instructions. The assay works by acetylcholinesterase converting the ACh present in the sample or standard to choline which is then oxidised to betaine and hydrogen peroxide (H_2O_2). When combined with horseradish peroxidase (HRP), H_2O_2 reacts with the Amplex Red reagent which produces a fluorescent product, resorufin.

Briefly, the samples were diluted with 1 X Reaction buffer and a positive control was made by diluting 20 mM H_2O_2 working solution with 10 µL of 1 X Reaction buffer. One hundred µL of both the samples and controls were added to each well on a 96 well plate. A working solution was made by mixing of 200 µL Amplex Red reagent, 100 µL HRP stock, 100 µL choline oxidase, 100 µL acetylcholinesterase and 9.5 mL of 1 X Reaction buffer. One hundred µL of the Amplex Red reagent/HRP/choline oxidase/acetylcholinesterase was added to each well containing samples and controls. The plate was then incubated,

while protected from light, for 30 minutes at room temperature. The fluorescence of the samples and standards were then measured by using a Modulus Microplate Multimode Reader using an excitation range of 530-660 nm and emission detection at approximately 590 nm. A standard curve of known ACh concentrations was generated using Prism 8 software **(Figure 2.7)**. Unknowns were interpolated using linear regression analysis.

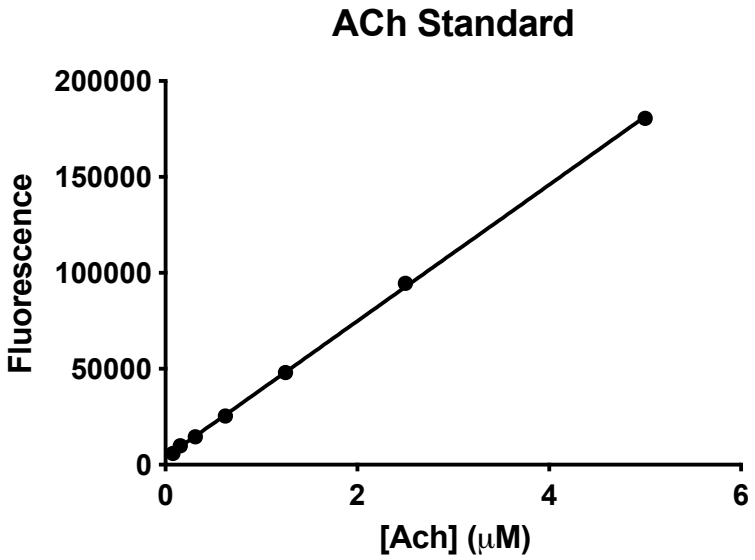

Figure 2.7: Representative ACh standard curve prepared using Amplex Red Ach assay kit, fluorescence analysed using a Modulus Microplate Reader, $r^2 = 0.9998$.

Quantification of ATP

ATP present in intraluminal and serosal samples was quantified using the ATP Luciferin/Luciferase ATP Determination Kit (Molecular Probes). This bioluminescence assay uses recombinant firefly luciferase and the substrate D-luciferin to determine ATP concentrations of the standards and samples. In the presence of ATP, luciferin converts to oxyluciferin, which produces light as a by-product.

Briefly, low-concentration ATP standard solutions were made by diluting 5 mM ATP in dH_2O. The standard reaction solution was made by mixing 8.9 mL dH_2O with 0.5 mL 20 X Reaction buffer, 0.1 mL 0.1 M DTT, 0.5 mL of 10 mM D-luciferin and 2.5 µL of firefly

luciferase. Ten µL of the standards and samples and 90 µL of standard reaction solution were added to each well on a 96 well plate. Luminescence was measured using a Modulus Microplate Reader with emission maximum of approximately 560 nm. ATP concentrations were quantified from a standard curve of known ATP concentrations and was constructed in Prism 8 software using linear regression analysis **(Figure 2.8)**.

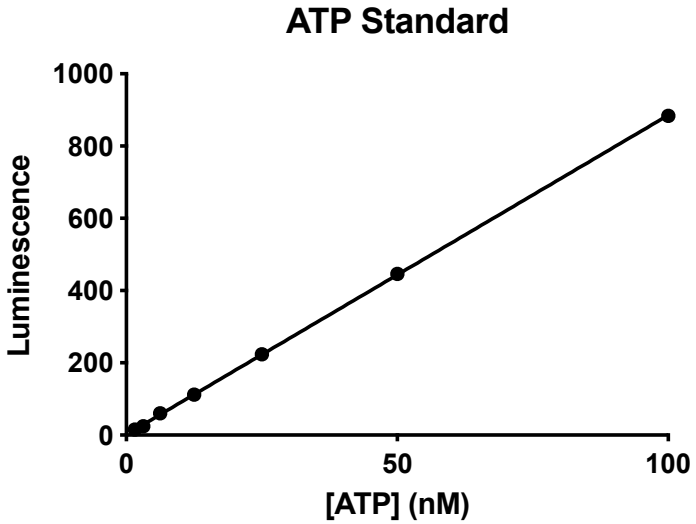

Figure 2.8: Representative ATP standard curve, measured using the ATP Determination Kit and luminescence analysed in a Modulus Microplate Reader, $r^2 = 0.9999$.

STATISTICS

All graphical analysis and statistical analysis were performed using GraphPad Prism 8 (GraphPad Software, San Diego, USA). Data is presented as mean ± standard error of the mean (SEM). All curve and time point analysis were undertaken using non-linear regression analysis to construct concentration-response and frequency-response curves, and curve fit analysis was used to compare curves between groups and generate EC_{50} or IC_{50} values. The EC_{50} value is the concentration of drug that gives half the maximal response while the IC_{50} value is the concentration of drug that inhibits the responses by 50%. Specific statistical analysis will be discussed in the following chapters. The null hypothesis was rejected when $p<0.05$.

SOLUTIONS

Krebs bicarbonate solution was made up as follows (mM). NaCl 118, $NaHCO_3$ 24.9, $CaCl_2$ 1.9, $MgSO_4$ 1.15, KCl 4.7, KH_2PO_4 1.15, and D-glucose 11.7. All reagents were obtained from Sigma Aldrich Co in St. Louis, USA. The carbogen gas (95% O_2 and 5% CO_2) used throughout the whole bladder preparations was obtained from BOC Gasses Australia.

DRUGS

All of the drugs used in the whole bladder preparation studies are listed below in **Table 2.1**. The concentrations of the drugs used in experiments were derived from concentrations used previously in the literature. All drugs were obtained from Sigma-Aldrich (SA) and Cayman Chemical Company (CCC).

Table 2.1: Pharmacological agents used in experiments

Compound	Main Action	Solvent	Cat No.
1-[3',4'-Dihydroxyphenyl]-2-isopropyl-aminoethanol (isoprenaline)	β-adrenoceptor agonist	H_2O	I5627
α,β-Methyleneadenosine 5'-triphosphate lithium salt (α,βmATP)	P2X receptor agonist	H_2O	M6517
Adenosine 5'-triphosphate disodium salt (ATP)	P2 purinoceptor agonist	H_2O	A7699
Atropine Sulfate Salt	Non-selective muscarinic antagonist	H_2O	A0257
Carbamylcholine chloride (carbachol)	Cholinergic receptor agonist (muscarinic >nicotinic)	H_2O	C240-9
Nω-Nitro-$_L$-Arginine (L-NNA)	Nitric oxide synthase inhibitor	H_2O	N-1522

CHAPTER 3: EFFECTS OF SOCIAL DEFEAT AND WITNESS TRAUMA ON BLADDER FUNCTION

The results presented in this chapter have been published: West EG, Sellers DJ, Chess-Williams R and McDermott C. (2020). "Voiding behaviour and efferent bladder function altered in mice following social defeat but not witness trauma". *Front. Physiol.* 11:247. doi: *https://doi.org/10.3389/fphys.2020.00247.*

INTRODUCTION

As covered in the previous chapter, psychological stress affects a large portion of society and is known to promote behavioural and psychological disturbances, in addition to affecting physical health (Schneiderman et al., 2005). In fulfilment of the overall aims of this thesis, two different stress models were used to determine the effects of different forms of psychological stress on bladder function. This chapter describes the social defeat and witness trauma mouse model which is clinically relevant as experiencing or witnessing traumatic events has been shown to increase the risk of developing anxiety, depression, and PTSD.

Socially Induced Stress: Social Defeat and Witness Trauma Model

The witness trauma and social defeat mouse model is a relatively new model representative of social stress, which aims to distinguish between the effects of physical (social defeat) and emotional (witness) social stress, while also introducing a component of social support (Li, Xu, & Wang, 2018). There are three main types of social stress (DeLamater & Ward, 2013);

- Abrupt and severe changes in life events
- Chronic strains which are defined as persistent events and
- Daily hassles which are minor events which do not require minimal adaptation

As the witness trauma and social defeat murine model experimental protocol occurs over a 10-day period, it is a protocol representative of chronic stress. Repeated exposure to stress leads to habituation in many animal models (Herman, 2013). Habituation occurs when the stressor becomes regimental and predictable. The rate of habituation is dependent on the type and severity of the stressor (Bhatnagar, Huber, Nowak, & Trotter, 2002). It can result in decreased responses to stressors upon repeated exposure leading to cumulative glucocorticoid secretion (Grissom & Bhatnagar, 2009). However, habituation is less likely to occur in the witness trauma and social defeat model as social stress has uncertain and unpredictable outcomes (Herman, 2013). These factors result in chronic stress leading to anxiety and depression.

There are several regions of the brain 'recruited' in the case of unpredictable social stress. This stress response is multifaceted and results in enhanced activity of the HPA axis. As discussed in the previous chapter, increased activity of the HPA axis leads to elevated glucocorticoid production and engagement of anxiety-like behaviours (Kinzig et al., 2003). There is also an increase in drive to the pre-frontal cortex of the brain which subsequently causes endocrine, behavioural and metabolic dysfunction (Flak, Solomon, Jankord, Krause, & Herman, 2012). This stress response is critical, however, when prolonged studies have associated this response with affective disorders such as depression and chronic anxiety (Kinzig et al., 2003).

Chronic stress disorders, such as anxiety, depression and PTSD have been linked to several different health problems. Comorbidity with other mental disorders, such as chronic anxiety and depression, is common in PTSD sufferers. One study in particular has observed that 90% of people with PTSD will have at least one lifetime comorbid mental disorder (Kessler, Sonnega, Bromet, Hughes, & Nelson, 1995). Several other studies have linked stress with bone and joint disorders, cardiovascular conditions,

neurological conditions, respiratory conditions and metabolic disease (Sareen et al., 2007).

Several clinical studies have investigated correlations between psychological stress and bladder pathologies. Stress appears to greatly influence or worsen symptom severity of bladder pathologies (Lai, 2015). Overactive bladder in particular has been linked to traumatic events in both childhood and adult life (Bradley, 2017). While this information gives an indication of the impact of psychological stress affecting the bladder, very little information is available on the exact mechanisms underlying this dysfunction.

Traditional rodent models of social stress use the subordination of one male by a larger, more aggressive male to simulate a socially-induced psychological stressor which leads to anxiety and depressive behaviour patterns (Golden, Covington, Berton, & Russo, 2011). An early study by Blanchard and Blanchard (1977) observed aggressive behaviour between 'intruder' rats and colonies of laboratory rats. The study found that when the male rat is placed with the aggressive rat, the subordinate male will assume supine posture and emit frequent calls of distress, which has been labelled as 'freezing behaviour'. The social defeat model is not only a test of physical stress but also measures the effect of continued psychogenic exposure after the protective barrier is placed between the dominant and submissive (Hollis & Kabbaj, 2014). Another early study investigated the acquisition of self-administered cocaine after social stress using *in vivo* micro dialysis. The study examined behavioural and dopaminergic responses, to social defeat, in previously exposed social defeat rats and rats that had never experienced social defeat. Rats that had previously been exposed to social defeat acquired cocaine self-administration in half the time of non-defeated rats (Tidey & Miczek, 1997). This study demonstrates persistent emotional stress that the social defeat paradigm may inflict. After exposure to the aggressor, submissive rats and mice exhibit elevated glucocorticoid

levels and hyperthermia, which is noted to take many hours to recover (Tornatzky & Miczek, 1993).

The social defeat studies mentioned above, however, do not consider the effect of indirect trauma on a witness. Many studies have found clear associations between direct exposure to a traumatic event and the development of PTSD, anxiety and depression (Patki, Salvi, Liu, & Salim, 2015). There has been less examination however, of the development of psychological disorders in persons with indirect exposure to trauma. Studies have found that among the specific types of traumatic exposure, sexual abuse, followed by exposure to crime and witnessing violence most frequently led to PTSD (Perrin et al., 2014). For this reason, a new model has been designed and implemented into recent studies (Sial, Warren, Alcantara, Parise, & Bolanos-Guzman, 2016). This model replicates both the social and physical stress in socially defeated mice and the emotional stress in witness mice, and when repeated over time to simulate chronic stress. There have been several studies using models of social defeat which have reported altered voiding behaviour of male rodents. Chang et al (2009) observed the voiding of both the dominant and submissive animals of a social defeat model and found that while voiding in the dominant animals remains unchanged, the submissive rodents develop characteristics of urinary retention (Chang et al., 2009). Similarly, Mann (2015) observed this trend with social defeat mice having a single large void. These changes in voiding behaviour were also accompanied by bladder wall hypertrophy, increased bladder capacity and the upregulation of CRF in Barrington's nucleus neurons (Wood, Baez, Bhatnagar, & Valentino, 2009). While these studies have been successful in assessing the voiding dysfunction of socially defeated mice, the underlying mechanisms associated with this dysfunction are still generally unknown. Also, the combined social

defeat/witness trauma model has never been used to investigate effects on bladder function.

Aims

The aim of the present study was to investigate the effects of a social defeat and witness trauma model on murine bladder function. Specific aims were:

- To investigate the effects of witness trauma and social defeat on voiding behaviour.
- To assess the effects of witness trauma and social defeat on serum corticosterone levels.
- To determine the effects of witness trauma and social defeat on local bladder function including compliance, contractile responses, and urothelial mediator release.

METHODS

Animals

Adult male C57BL/6J (12-14 weeks old) mice were used as social defeat and witness animals, while ex-breeder ARC(S) mice were used as aggressors. All mice were obtained and housed as outlined in the general methodology chapter (Chapter 2) randomly allocated into one of three groups: (1) Control/Unstressed, (2) Social defeat and (3) Witness, with n=6 animals included in each group.

Screening of Aggressors

Male ex-breeder ARC(S) mice were housed individually and screened for persistent aggressive behaviour using test C57BL/6J mice prior to experimental work. The screening protocol is one that has previously been described using two different types of aggressor strains, ARC(S) and CD1 (Keeney et al., 2006; Sial et al., 2016). Due to availability, ARC(S) ex-breeders were used for the experimental protocol. Screening involved placing an ARC(S) and test C57BL/6J mouse in the plexiglass chamber shown in **Figure 3.1**. The removable panel was lifted, and initial time to attack was recorded within a maximum 5-minute period. To be labelled aggressive, aggressor mice had to attack within the first 60 seconds of interaction for 3 consecutive days, over a 5-day period (Sial et al., 2016). Of 10 aggressive mice, 4 were labelled as aggressive and used for the following stress protocol.

Witness Trauma/Social Defeat Stress Protocol

A joint model of social defeat (physical stress) and witness trauma (emotional stress) was employed using a variation of the methods previously described (Golden, Covington, Berton, & Russo, 2015; Li et al., 2018; Sial et al., 2016).

Adult male C57BL/6J mice (12-14 weeks) were housed together in pairs for 3-days prior to and during the 10-day stress protocol, to allow for bonding. The mice were randomly allocated to either the social defeat or witness trauma experimental group. C57Bl/6J pairs were placed into the custom-made plexiglass chamber with one ARC(S) aggressor mouse for 1 hour per day for 10-days. The witness mouse was physically separated from the other mice during the 1-hour stress protocol by a transparent perforated wall but could observe interactions between its cage mate and the aggressor ARC(S) mouse. The social defeat mouse was in physical contact with the aggressor ARC(S) for a maximum of 5 minutes. After 5 minutes, the ARC-1 mouse was separated from the social defeat animal by a transparent perforated barrier for 55 minutes, so the mice could still smell and see each other. This protocol was repeated for 10 consecutive days with Voiding Pattern Analysis taking place on days 0, 1, 3, 5, 7 and 10 as described in Chapter 2.

To maintain aggression, aggressor mice were used in rotation, so social defeat mice did not encounter the same aggressor on consecutive days. Several aspects of aggressive behaviour were recorded, including latency to attack (seconds) and how many bouts of aggression occurred. A 'bout' of aggression was defined as grabbing and pulling, biting and following or chasing. It was also noted if the aggressors drew blood and at what time. To minimize risk of wounding, all social defeat sessions were observed continuously, and the animals separated if the social defeat mouse exhibited clear submissive behaviour, including submissive posture or freezing. If mice were defeated before the 5-minute interaction period ended, mice were separated early using the plexiglass barrier shown

in **Figure 3.2**. Wounding, but not superficial scratches, is associated with innate immune response (Foertsch et al., 2017) and through all the observed defeat sessions, no social defeat animals received wounds requiring their exclusion from the experiment. Age matched control C57Bl/6J pairs were housed under normal conditions for the duration of the study.

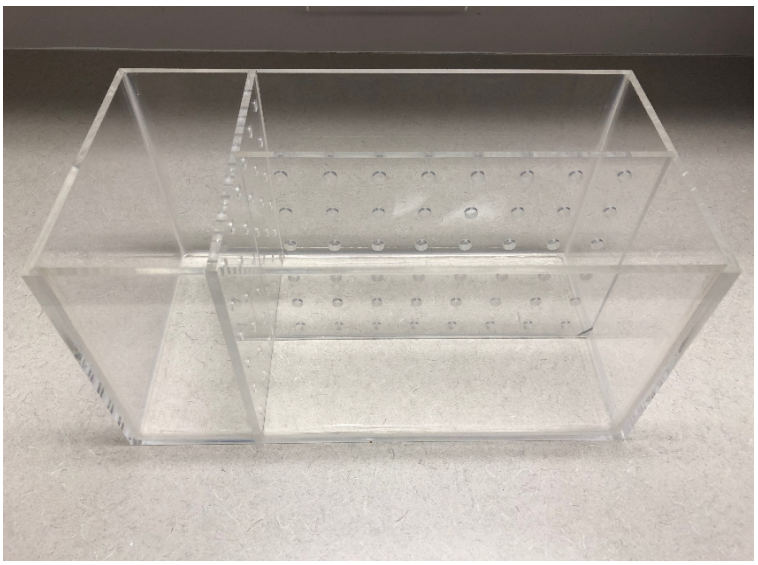

Figure 3.1: Photograph of witness trauma and social defeat chamber (Photograph taken by the author).

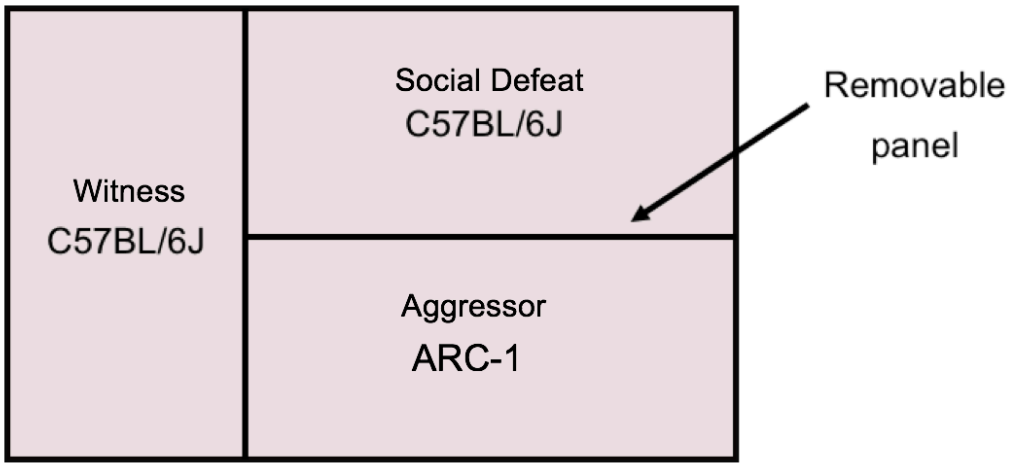

Figure 3.2: Diagram of social defeat and witness trauma chamber (Image created by the author).

Whole bladder preparations

Whole bladder preparations were performed as described in Chapter 2, with the following differences due to sex of mice. When the abdominal region was secured to the dissection bath under the dissection microscope, male reproductive organs were removed including the testes, prostate and surrounding connective tissue. The whole bladder preparation then continued as described in Chapter 2.

Statistical Analysis

Two-way ANOVA with Tukey's post hoc test for multiple comparisons was used to compare the different time points and voiding variables. The same statistical tests were also used to analyse the concentration-response curves. One-way ANOVA with Bonferroni post-hoc test was used to compare differences between the three animal groups (urothelial mediator release data, animal parameters, spontaneous contractile activity data, contractile responses to ATP and KCl).

RESULTS

Animal Parameters and Voiding Behaviour

During the stress exposure period, animal body weight and water consumption was measured on day 0 to obtain baseline data shown below **(Table 3.1)**. The body weight and water consumption of mice was also measured on day 1, 3, 5, 7 and 10. These parameters were unchanged throughout the stress protocol and neither the social defeat or witness trauma stress affected the body weight or water consumption over time.

Bladder weight was measured at the end of the whole bladder preparation protocol and there was no difference between the unstressed, social defeat or witness groups as seen below **(Table 3.1).**

TABLE 3.1: *Baseline body weight, water consumption and bladder weight (at day 10) following stress protocol in control (unstressed), social defeat and witness mice.*

	Unstressed	Social Defeat	Witness
Body weight (g)	26.30 ± 0.67	26.70 ± 0.68	26.30 ± 1.04
Bladder weight (mg)	22.30 ± 1.18	21.50 ± 0.49	22.96 ± 0.21
Water consumption (g)	0.64 ± 0.19	0.79 ± 0.23	0.98 ± 0.66

At the time of euthanasia, a blood sample was taken for plasma corticosterone analysis. **Figure 3.3** below shows a plasma corticosterone level of 24.90 ± 1.93 ng/mL in control (unstressed) mice, 54.70 ± 4.87 ng/mL in social defeat mice ($p = 0.0001$) and 44.86 ± 4.66 ng/mL in witness mice ($p = 0.0037$). This significant difference indicated that a hormonal stress response was present in both social defeat and witness groups.

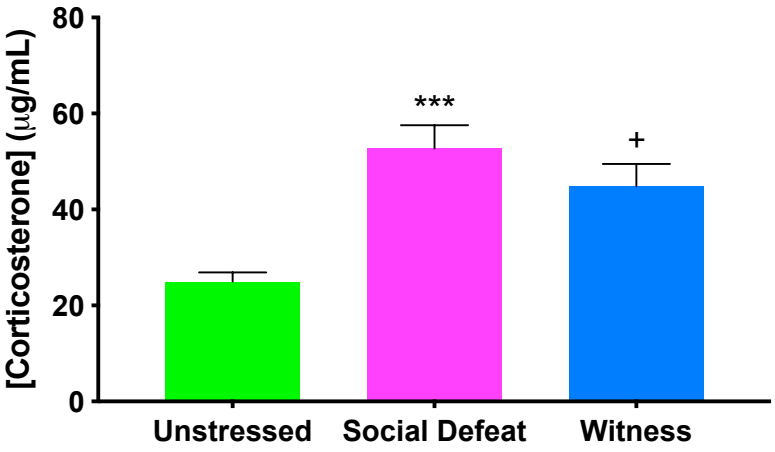

Figure 3.3: Plasma corticosterone levels in mice from unstressed, social defeat stress and witness groups. Datum is represented as mean ± SEM (n = 7). Analysis was performed by a one-way ANOVA (* $p < 0.001$, unstressed vs. social defeat) ($^{+}p < 0.05$, unstressed vs. witness).**

Voiding pattern analysis was undertaken, as detailed in Chapter 2, in all three animal groups to examine changes in voiding behaviour.

Overall, there was no significant difference in total void area between the three animal groups over time, which indicates that urine production was not affected by social defeat or witness trauma stress **(Figure 3.4.C)**. There was, however, a significant decrease in urinary frequency **(Figure 3.4.A)**, between the social defeat group and both witness and unstressed control groups. This change was significantly evident following day 3 ($p = 0.027$) and day 7 ($p = 0.019$) of stress exposure. Although variable across the 10-days of stress, average void size was significantly increased at both day 3 ($p = 0.0296$) and day 7 ($p = 0.0019$) in the social defeat group compared to the unstressed controls at day 7 ($p = 0.0168$) in the social defeat group compared to the witness. This fits with **Figure 3.4.A** in that when the number of voids decreases, the average void size increases, meaning that the social defeat mice are voiding less frequently, and each void is larger than in controls. The number of small voids less than 0.2 cm^2 **(Figure 3.4.D)**, was decreased in the social defeat, although not significantly, compared to the control and witness groups. The

absence of changes in both water consumption and voided volume indicates that the change observed is an actual change in urinary frequency and not reduced urine production. Voiding behaviour was not significantly altered in the witness group.

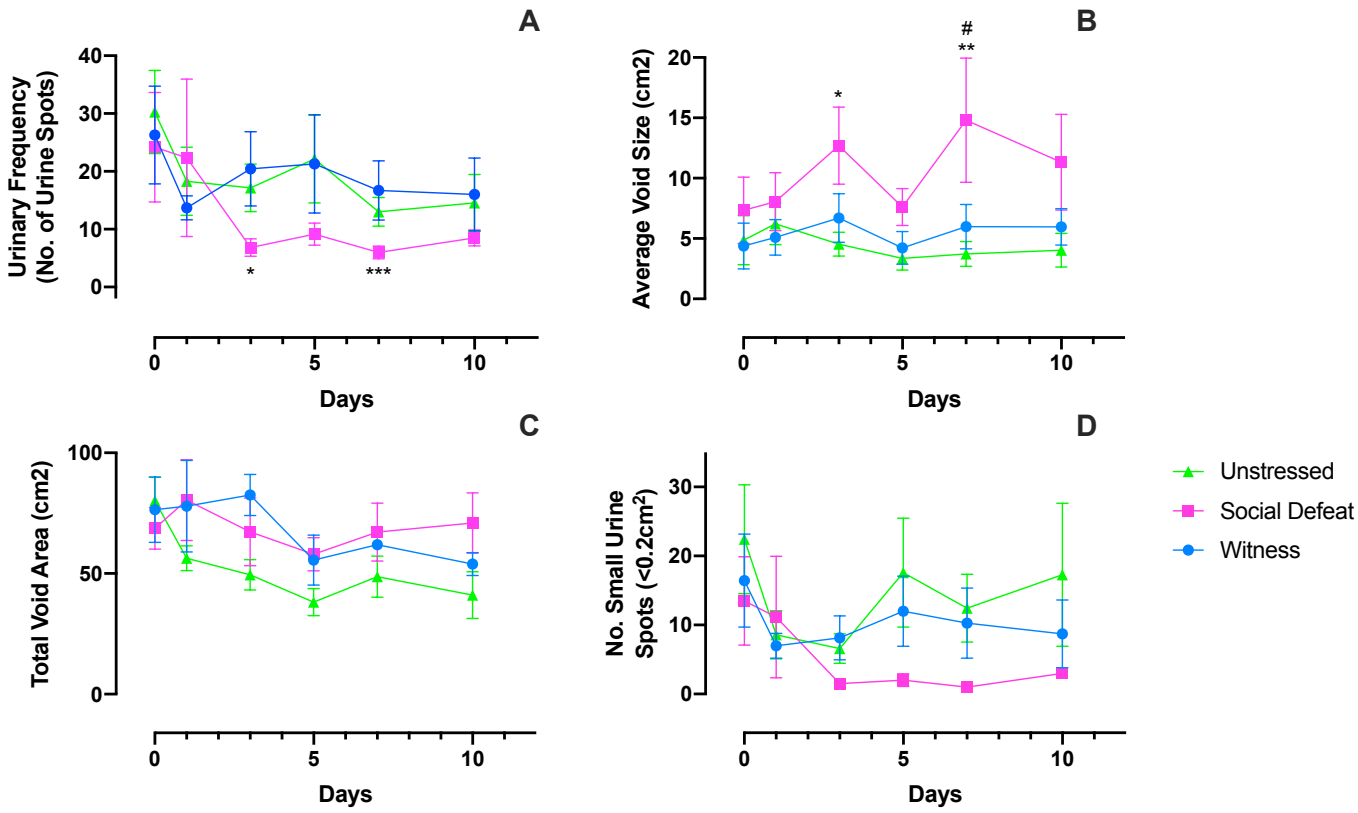

Figure 3.4: Voiding pattern analysis conducted in mice from unstressed, social defeat stress and witness groups. (A) Number of voiding events, (B) Average voided area, (C) Total voided area and (D) Number of small urine voids smaller than 0.2cm². Datum is presented as mean ± SEM (n = 7). Analysis was performed using two-way repeated measures ANOVA (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, unstressed vs. stressed) (#$p < 0.05$, stressed vs. witness).

At the time of filter paper collection after the 4-hour voiding pattern analysis period, faecal pellets were collected from the voiding paper and left to dry overnight to be weighed the next day. There was no significant difference in either the number **(Figure**

3.5.A) or weight **(Figure 3.5.B)** of the pellets between the social defeat, witness and unstressed control group.

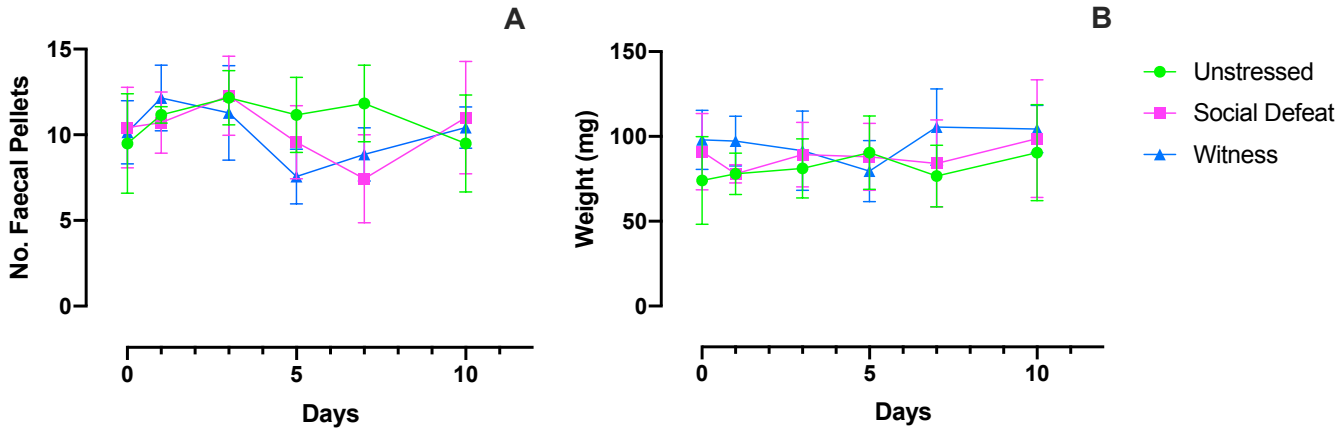

Figure 3.5: Faecal pellet analysis conducted in mice from unstressed, social defeat stress and witness groups. (A) Number of faecal pellets, (B) relative weight of faecal pellets. Datum is presented as mean ± SEM (n = 7). Analysis was performed using two-way repeated measures ANOVA.

Mediator Release

The intraluminal fluid, taken from distended bladders at 20mmHg, and the serosal fluid, taken from the bath were analysed for ATP and ACh content. The concentration of each mediator in the individual samples was calculated and normalised using intraluminal and serosal volume to determine the total amount present. Both intraluminal and serosal release of ATP were not altered in social defeat or witness trauma animals, nor was serosal release of ACh **(Figure 3.6.A, B & D)**. However, total release of ACh into the bladder lumen was greater, and borderline significant, in the social defeat group (1.13 ± 0.16 nmoles n=7) when compared to controls (0.73 ± 0.17 nmoles n=7), although this change did not meet statistical significance ($p = 0.05$) **(Figure 3.6.C)**.

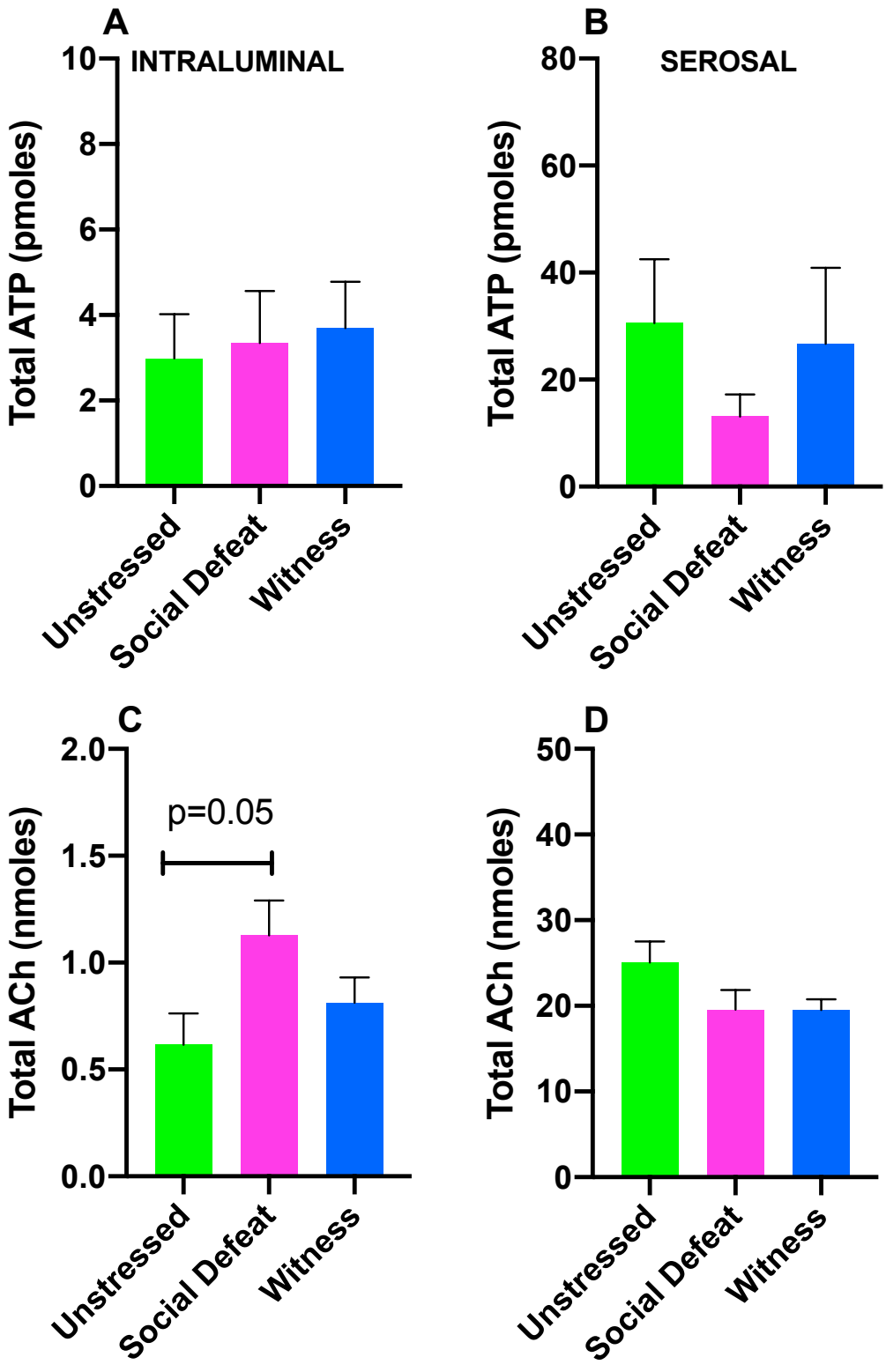

Figure 3.6: Release of ATP and ACh into the (A & C) intraluminal and (B & D) serosal fluid collected following distensions of isolated bladders from unstressed, social defeat stress and witness groups. Datum is represented as mean ± SEM (n = 7). Analysis was performed as a one-way ANOVA.

Bladder Compliance and Stretch-relaxation

As a measure of compliance, the volume-pressure relationship of whole bladder was assessed during filling with Krebs-bicarbonate solution to a physiological pressure of 20 mmHg. Bladder compliance was not significantly changed in the social defeat or witness groups compared to the unstressed controls **(Figure 3.7).**

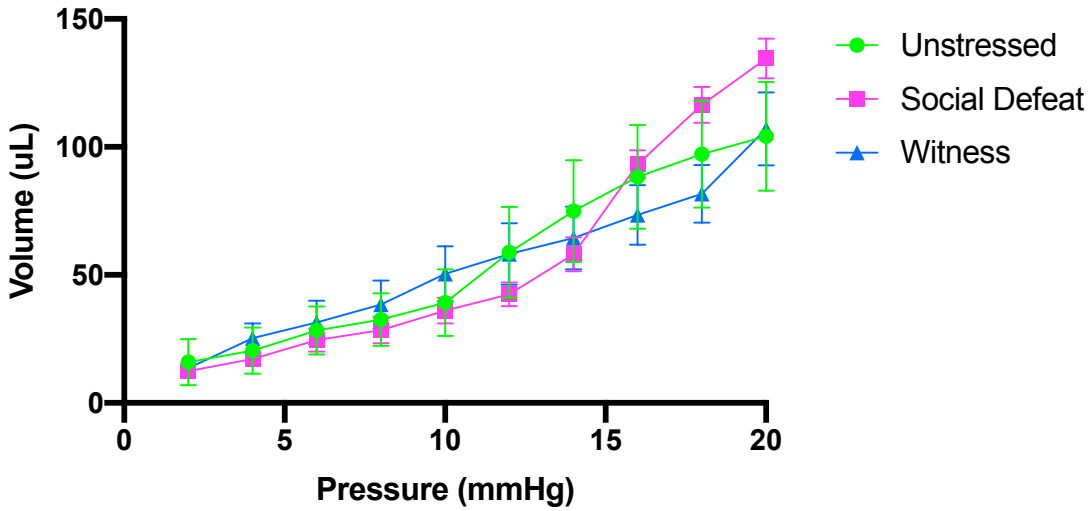

Figure 3.7: Volume-pressure relationship for bladders from unstressed, social defeat stress and witness groups. Datum is represented as mean ± SEM (n = 7), analysed by two-way ANOVA.

To measure, bladders were infused with Krebs-bicarbonate solution to a pressure of 20 mmHg at which point the perfusion pump was stopped and outflow tap remained closed. The bladders were left for 30 minutes to equilibrate and allow for the muscle to stabilise. Over the course of 30 minutes, all the bladders relaxed from their initial pressure and plateaued. The pressure-time relationship was then assessed to observe overall stretch-relaxation across the 3 animal groups **(Figure 3.8)**. The sharpest decreases in response to stretch occurred between 0 and 5 minutes, and therefore, the 'X' axis on the graph is

extended to highlight this period. There was no significant difference between any of the groups.

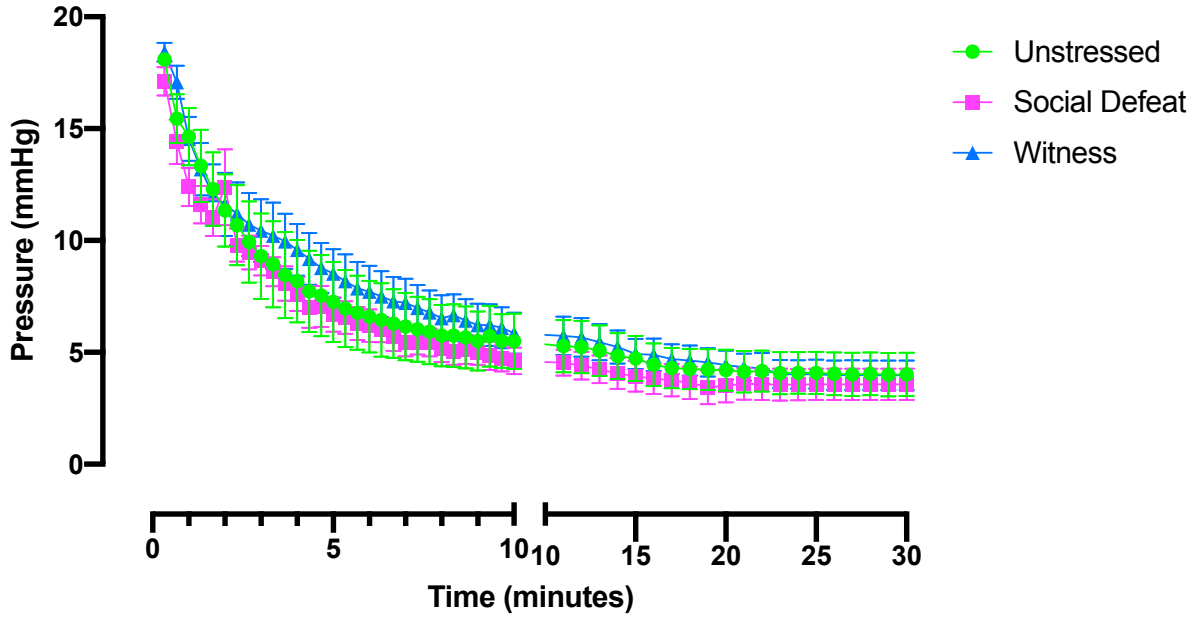

Figure 3.8: Pressure-time relationship for bladders from unstressed, social defeat stress and witness groups after filling to an initial pressure of 20 mmHg. Datum is represented as mean ± SEM (n = 7), analysed by two-way ANOVA.

Bladder Contractility and Response to Electrical Field Stimulation

There was no significant change in the contractile response to KCl (60 mM) across the Unstressed, social defeat and witness groups with mean pressure changes recorded as 31.90 ± 5.48 mmHg, 30.28 ± 5.22 mmHg and 31.30 ± 2.44 mmHg (n=7), respectively **(Figure 3.9)**.

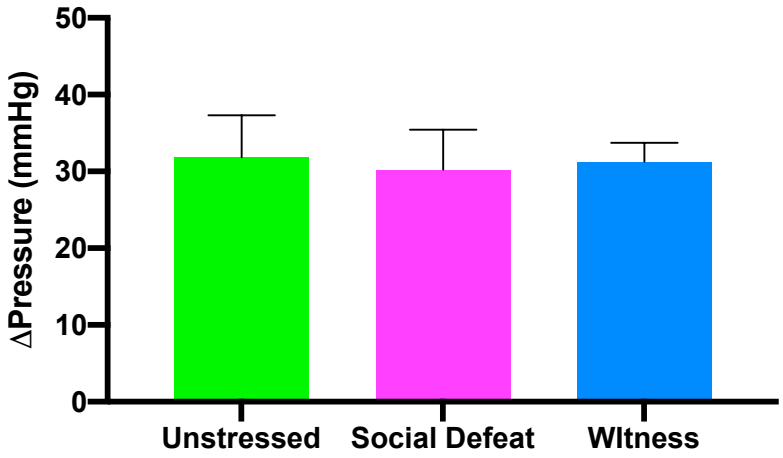

Figure 3.9: Pressure responses to KCl (60 mM) in bladders from unstressed, social defeat stress and witness groups. Datum is represented as mean ± SEM (n = 7) and analysed using an Ordinary one-way ANOVA with Tukey Analysis.

Increasing frequencies of electrical stimulation elicited frequency-dependent increases in pressure in all three groups. The contractile responses to EFS were significantly enhanced at 5, 10 and 20 Hz ($p = 0.004$, $p = 0.001$, $p = 0.0001$ respectively) in bladders from the social defeat animals compared with the control and witness. Responses of bladders from witness animals to EFS were unchanged as can be seen in **Figure 3.10** below.

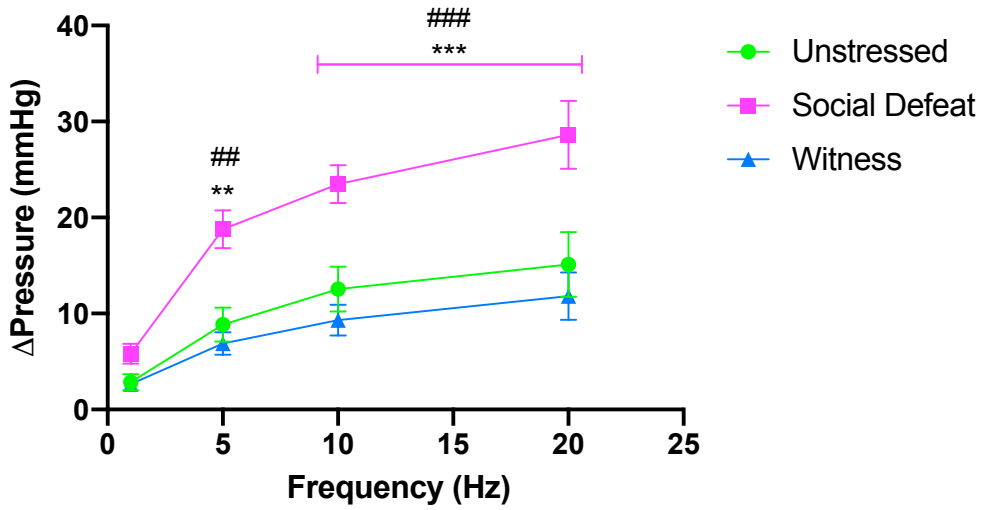

Figure 3.10: EFS-evoked contraction of isolated bladders from unstressed, social defeat stress and witness mice, at 1, 5, 10 and 20 Hz. Responses were recorded as a change in pressure from baseline. Datum was analysed using two-way repeated measures ANOVA with Tukeys multiple comparison ($p < 0.01$, ***$p < 0.001$, social defeat vs. witness) (\#\#$p < 0.01$, \#\#\#$p < 0.001$, social defeat vs. witness).**

Responses to EFS were repeated at 20 Hz in the absence and presence of several pharmacological agents to determine to relative contribution of NO, ACh and ATP to the responses.

Addition of the NOS inhibitor LNNA (100 μM) did not significantly alter bladder contraction to EFS in any of the groups, indicating that the inhibitory neurotransmitter NO was not influencing neurotransmission **(Figure 3.11.A).** Addition of the muscarinic antagonist atropine (1 μM) reduced the responses to 20 Hz EFS by 48.50 ± 7.99% in unstressed bladders, this change was lower in the social defeat and witness group, although not significantly so (33.30 ± 9.39% and 32.30 ± 8.67% respectively, n=7) **(Figure 3.11.A and B)**. Desensitization of purinergic receptors with αβmATP (1 mM) reduced the response to EFS to a similar extent in bladders from each group (unstressed 41.90 ± 6.53%; social defeat 54.80 ± 8.55%; witness 43.20 ± 13.60%, n=7), with no significant difference between the groups detected **(Figure 3.11.A and C)**. This

demonstrates that while nerve-evoked contractions were enhanced by social defeat, the relative contribution of ACh and ATP was unchanged.

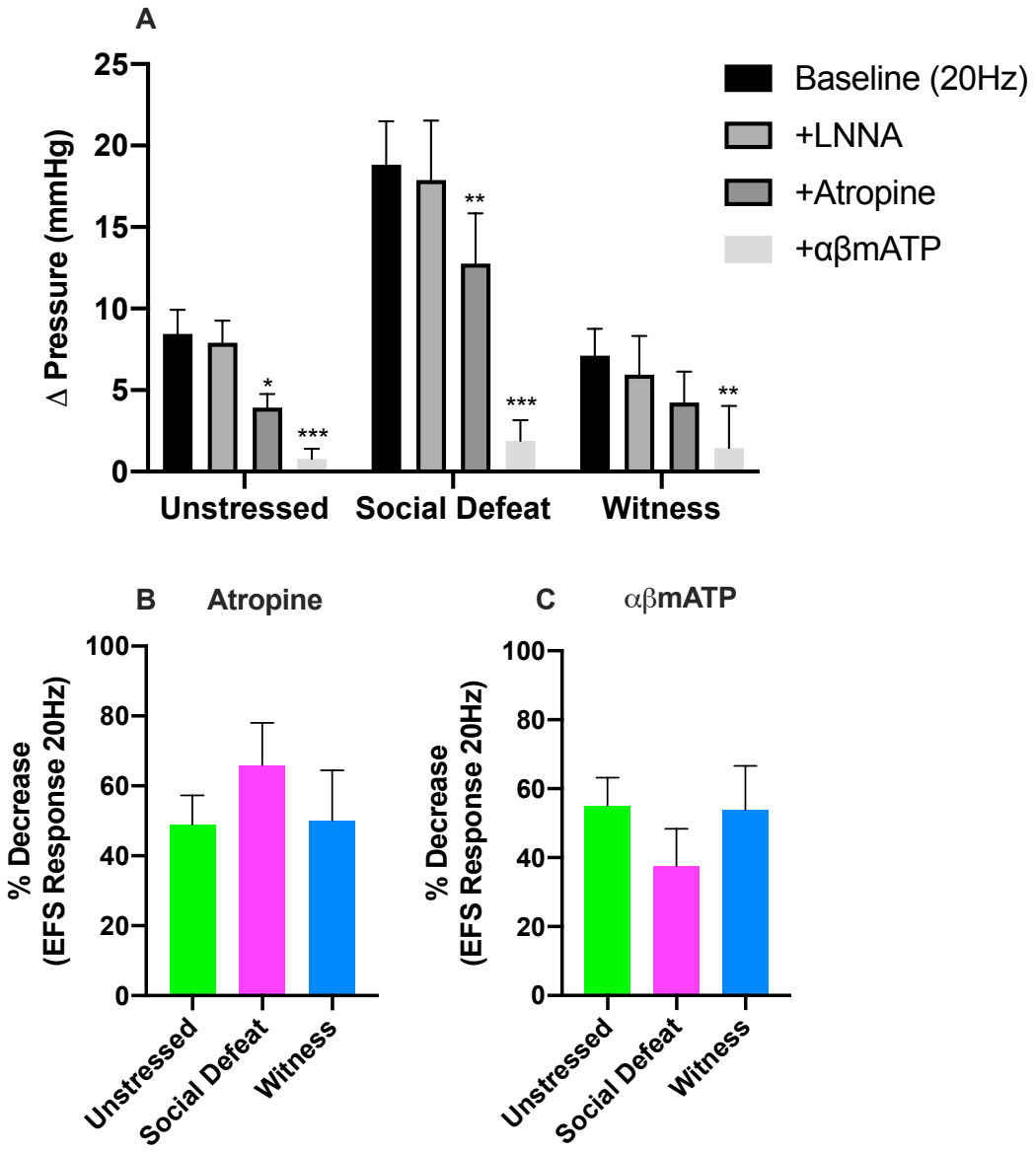

Figure 3.11: (A) Intravesical pressure responses to electrical field stimulation at 20 Hz (baseline), and after addition of L-NNA (100 μM), atropine (1 μM) and αβmATP (1 mM) to bladders from unstressed, social defeat stress and witness mice. Percentage decrease in EFS response on addition of (B) atropine and (C) αβmATP to bladders from each group. Datum is expressed as mean ± SEM and analysed using two-way repeated measures ANOVA with Dunnett's multiple comparisons test for (A) and one-way ANOVA with Dunnett's multiple comparisons test for (B and C) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. Baseline (20 Hz)).

Responses to Pharmacological Agents

Contractile responses of isolated bladders were observed following addition of several pharmacological agents. These pharmacological agents were added to the serosal fluid and pressure responses were recorded as changes from baseline. Purinergic stimulation was assessed using ATP (10 mM) and αβmATP (1 mM). Responses to ATP **(Figure 3.12.A)** were not significantly different across the unstressed, social defeat and witness groups (13.61 ± 6.28 mmHg, 10.42 ± 2.36 mmHg and 8.09 ± 2.02 mmHg respectively, n=7).

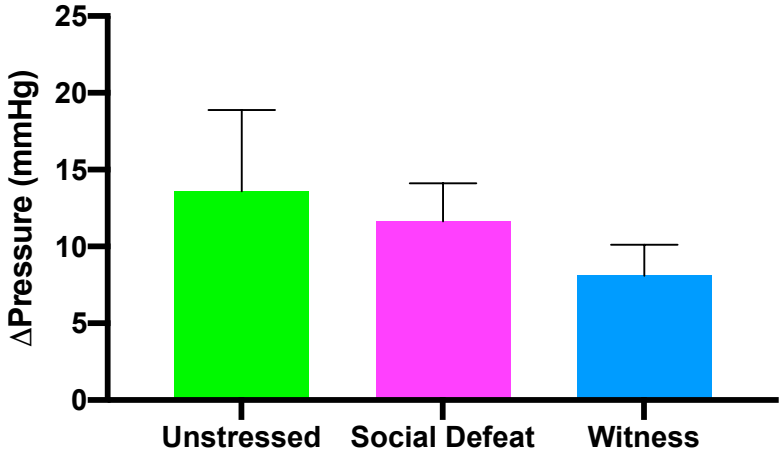

Figure 3.12: Pressure responses to ATP (10 mM), in isolated bladders from unstressed, social defeat stress and witness mice. Datum is represented as mean ± SEM (n = 7) and analysed using an ordinary one-way ANOVA with Tukeys comparison.

The contractile response to αβmATP (1 mM) was also recorded **(Figure 3.13)**. The responses displayed in the unstressed group (13.80 ± 1.84 mmHg, n=7) showed no significant differences when compared to the social defeat group (19.20 ± 4.05 mmHg, n=7) and the witness group (7.88 ± 1.19 mmHg, n=7). However, there was a significant difference in responses between the social defeat stress and witness group ($p = 0.0156$).

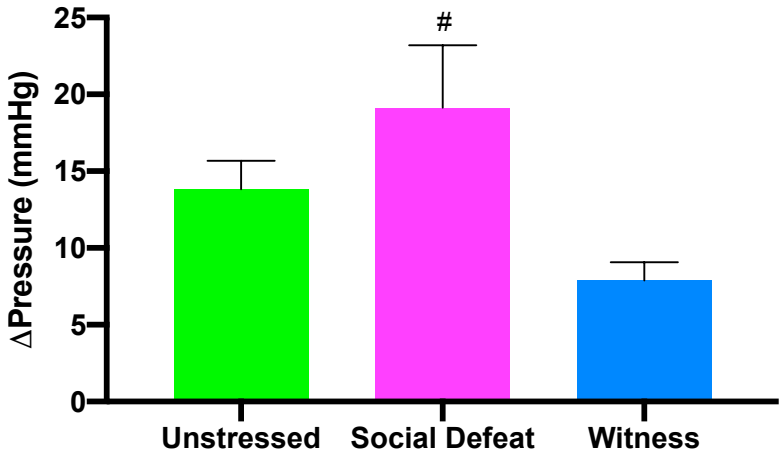

Figure 3.13: Pressure responses to $\alpha\beta$mATP (1 mM) in bladders from unstressed, social defeat stress and witness mice. Datum is represented as mean $\pm$ SEM (n $=$ 7) and analysed using an ordinary one-way ANOVA with Tukey comparison ($^{\#}p < 0.05$, stressed vs. witness).

Following washout to baseline, a cumulative concentration-response curve to carbachol was obtained on each isolated bladder to study the contractile response to muscarinic stimulation **(Figure 3.14)**. A two-way ANOVA with Tukey multiple comparisons test revealed no significant difference in the responses to carbachol between groups **(Figure 3.14)**. There was no significant difference in either pEC_{50} values or maximal responses **(Table 3.2)**. The concentration response data was also expressed as a percentage of the KCl response, with no significant difference observed between the groups.

TABLE 3.2: *Whole bladder responses to carbachol in unstressed controls, social defeat and witness mice (n=7).*

	Unstressed	**Social Defeat**	**Witness**
pEC_{50}	6.19 ± 0.08	6.04 ± 0.10	6.01 ± 0.09
Maximal response ΔPressure (mmHg)	42.79 ± 3.92	36.66 ± 3.91	43.92 ± 5.01

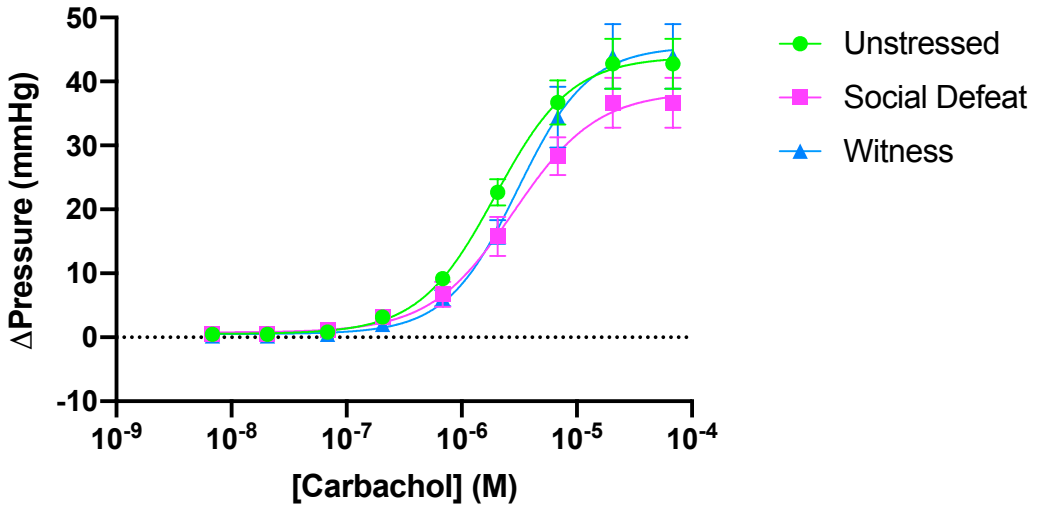

Figure 3.14: Carbachol concentration-response curves, for isolated bladders from unstressed, social defeat stress and witness mice, recorded as change in intravesical pressure from baseline. Datum is represented as mean ± SEM (n = 7), analysed using non-linear regression and two-way ANOVA with Bonferroni's multiple comparisons test.

One μM carbachol was added to the bath to pre-contract the bladder prior to measurement of relaxation to cumulative concentrations of isoprenaline (β-adrenoceptor agonist). No change was evident in the maximal response or the pIC_{50} values **(Table 3.3)**.

TABLE 3.3: *Whole bladder responses to isoprenaline in unstressed control, social defeat and witness mice (n=7).*

	Unstressed	Social Defeat	Witness
pIC_{50}	6.65 ± 0.18	6.73 ± 0.31	6.65 ± 0.17
Maximal response			
ΔPressure (mmHg)	- 6.31 ± 0.78	-4.34 ± 0.35	-5.26 ± 0.92
Response (% Decrease)	5.14 ± 12.42	-17.53 ± 22.05	-4.37 ± 18.52

Relaxations to isoprenaline were similar in all three groups when responses were expressed as a percentage of the carbachol pre-contraction. There was, however, a significant difference in maximum relaxation to isoprenaline in the social defeat mice compared to the unstressed controls at higher doses (*$p = 0.01569$ and **$p = 0.0049$).

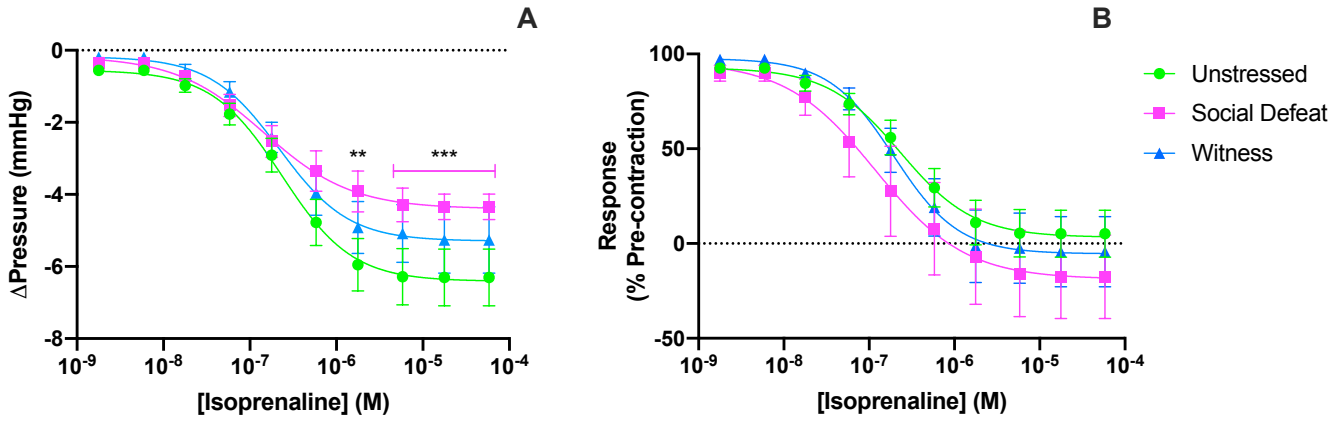

Figure 3.15: Effect of isoprenaline on isolated bladders, from unstressed, social defeat stress and witness mice. (A) Raw datum as fall in pressure from pre-contraction level, (B) Relaxation as a percentage (%) of the carbachol precontraction. Datum is represented as mean ± SEM (n = 7), using two-way ANOVA and non-linear regression ($p < 0.01$, ***$p < 0.001$, stressed vs. unstressed).**

Spontaneous Phasic Contractions

During experiments, phasic activity was assessed twice; during bladder stretch-relaxation and during the pre-contraction before isoprenaline responses (i.e., in the presence of carbachol (1 μM)). The first was spontaneous contractions of the bladder during the stretch-relaxation period from an elevated intraluminal pressure. The average frequency **(Figure 3.16.A)** and the amplitude **(Figure 3.16.B)** of the spontaneous activity compared to the unstressed mice was increased in bladders from social defeat mice, however this was not a statistically significant change. Witness mice had similar spontaneous activity compared to the unstressed control mice.

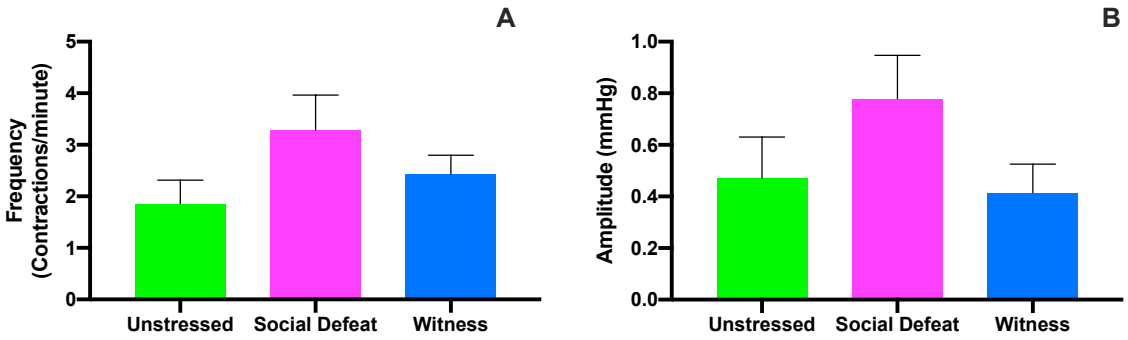

Figure 3.16: Spontaneous phasic contractions during stretch-relaxation from unstressed, social defeat stress and witness mice. (A) Frequency of contractions/minute and (B) Amplitude of spontaneous contractions. Datum is represented as mean ± SEM (n = 7), analysed using one-way ANOVA with Tukey's multiple comparison.

The second period of phasic activity was measured following stimulation of a tonic contraction with 1 μM carbachol. The phasic contractions were measured during the carbachol pre-contraction plateau. There was no significant difference in either the tonic contraction, the frequency **(Figure 3.17.A)** or amplitude **(Figure 3.17.B)** of the phasic contractions at the plateau, across any of the animal groups.

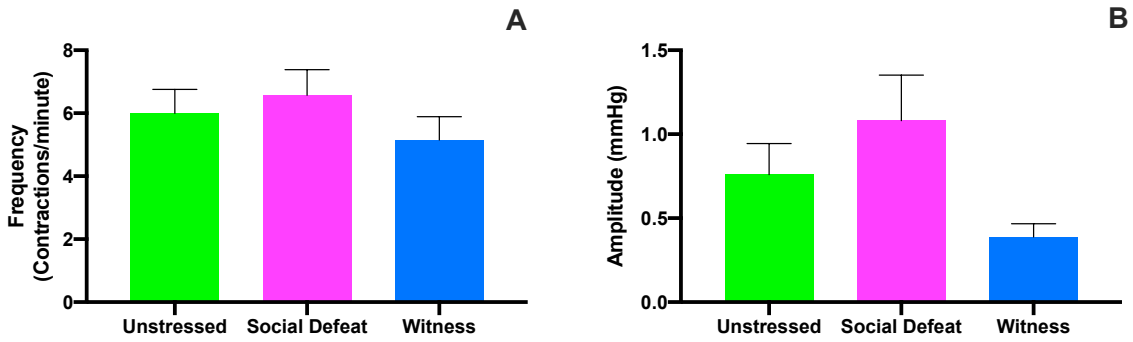

Figure 3.17: Phasic response to 1 μM carbachol measured in isolated bladder from unstressed, social defeat stress and witness mice. (A) Frequency of phasic contractions and (B) Amplitude of phasic contractions. Datum is represented as mean ± SEM (n = 7), analysed using a one-way ANOVA with Tukey's analysis.

DISCUSSION

An animal model of physical and emotional social stress was employed to determine what impact social defeat and witness trauma stress have on the bladder, and if both types of stressor affect the bladder in the same way.

Effects of Stress on Voiding Behaviour

Despite hormonal changes being similar in both the witness and social defeat mice, voiding frequency was only reduced in the social defeat mice, compared to the control. This voiding dysfunction has also been observed in a number of other studies using the same model (Chang et al., 2009; Mann, 2015).

The study by Mann (2015), compared the altered bladder function induced by two types of stress, social defeat stress and chronic restraint stress. The study showed a similar increase in plasma corticosterone levels in both animal groups, as seen in our model. When analysing voiding pattern, Mann (2015) found that the social defeat mice performed a large single void compared to multiple smaller voids exhibited by the control mice. The chronic restraint stressed mice however showed the same voiding pattern as the control mice. This is similar to the witness mice in the present study, where no change in voiding behaviour was observed. The reason for this may come back to the social support theory discussed above and by Li et al. (2018). The study found that emotionally stressed (witness) adolescent mice benefited from the social support gained by being housed, as a pair, with the physically stressed mice (social defeat) after aggressor interaction. The physically stressed mice exhibited impairment of social interest which would suggest that this stressed group is less capable of effectively seeking and using social support. This may explain why, despite the similarly raised plasma corticosterone levels seen in our study, voiding changes are less evident in witness mice.

While voiding frequency of the social defeat mice was decreased, the average void volume increased. However, as the total voided volume did not change between groups, water consumption can be ruled out as the cause of this dysfunction. Several studies have observed the same trend in their social defeat mice (Chang et al., 2009; Mann, 2015; Wood et al., 2009), however, the underlying mechanism of this urinary retention phenotype has never been discussed in detail. Several studies have observed the effect of social defeat stress on the HPA axis and Barrington's nucleus (pontine micturition centre) in relation to hormone production and the effect on the micturition reflex.

A study by Keeney et al. (2006), collected the brains of mice which had undergone both acute and chronic social defeat stress. The brains were then sectioned, and hybridisation was performed in order to detect CRF mRNA and AVP (arginine vasopressin) mRNA. The study found that CRF mRNA was upregulated after acute stress while AVP mRNA was upregulated in chronic social defeat mice. The study by Keeney et al. (2006) also associated increased AVP mRNA with a selective increase in AVP peptide hormone production and release into the portal system. AVP is primarily responsible for maintaining body fluid homeostasis and acts on three different receptors; V_{1a}, V_{1b} and V_2 (Pisipati & Hashim, 2011). AVP receptors are most commonly associated with blood vessel constriction and fluid reabsorption in the kidneys and is known to be a powerful vasoconstrictor. There have been studies, however, which have identified and characterised AVP receptors in the rat urinary bladder. One study used saturation binding experiments with radioactive AVP to identify one class of high-affinity, low-capacity binding sites in the bladder (Thibonnier, Snajdar, & Rapp, 1986). The study then used receptor specific antagonists to differentiate between the V_1 and V_2 AVP receptors and concluded that V_1 AVP receptors were the main receptor in the rat urinary bladder (Thibonnier et al., 1986). A later study observed the effect of *in vivo* versus in vitro AVP

on normal and obstructed rat urinary bladder. The study found that contractility of the detrusor muscle was greater in the normal bladder treated with AVP compared to the obstructed bladder (Berggren, Andersson, Lundin, & Uvelius, 1993). While these studies have been primarily performed on rats due to the ease of working with a larger brain, they indicate that AVP may act on V_1 receptors on the bladder, thereby increasing contractile ability to empty the bladder on a single void, producing the urinary retention phenotype observed in our study.

In terms of CRF, a study by Wood et al. (2009), found that chronic social defeat of male rats also caused a urinary retention phenotype. The study looked at the brains of stressed rats, 24 hours after the final stress exposure, and found that social defeat increased expression of CRF in the Barrington's nucleus. CRF has been shown to have an inhibitory influence on the spinal projections of the Barrington's nucleus, which regulate the micturition reflex (Pavcovich & Valentino, 1995). CRF expression was also observed to increase in the bladder and serum after psychological stress, which suggests central changes are also at play (Seki et al., 2019).

Effects of Stress on Corticosterone Release

Exposure to physical (social defeat) and emotional (witness) stress produced a similar hormonal stress response in both animal groups. This confirmed that the mice used in this study were stressed when compared to controls. Many experimental studies looking at both the social defeat (Mann, 2015) and social defeat/witness model (Li et al., 2018; Sial et al., 2016) have also reported increased corticosterone levels in their animals.

As stated in the methodology, plasma samples were collected 24 hours after the final stress exposure. Several studies of social defeat and witness trauma have also observed the long-term release of corticosterone. One study measured plasma corticosterone

levels of both the witness and defeated mice 40-minutes after the first stress exposure and 24-hours after the 10th day of stress. This method allowed the group to compare the hormonal response to acute versus chronic stress. Both witness and social defeat stress exposure significantly raised corticosterone levels after acute stress. After 10-days of stress exposure corticosterone levels were further increased, although the levels in witness mice were never as high as in social defeat mice (Warren et al., 2013). This correlates with the data presented in this chapter where corticosterone levels of witness mice were still significantly increased compared to the control but still not as high as in the social defeat mice.

Increased corticosterone has been linked to hyperactivity of the HPA axis. This hyperactivity has been implicated in the pathogenesis of both anxiety and depression in humans (Holsboer, 2001). A previous study looking at a murine social defeat model observed the same trend in corticosterone levels, over four, eight and twelve stress exposures. The study found enhanced corticosterone release after twelve stress exposures as opposed to eight and four. The study concluded that the HPA axis stress response appears to adapt to short-term acute stress, however after chronic stress it becomes maladaptive (Keeney et al., 2006). The study also found that the corticosterone response is also dependent on the where the subordinate and dominate mice are housed. Another study by the same group found that living in close proximity to the dominant mouse may lead to an 'anticipatory' stress of the HPA axis which may be as stimulating as actual perceived stress (Keeney & Hogg, 1999). This indicates the importance of housing of animals when considering the social defeat model. Housing of witness and social defeat mice has also been examined in other studies. A study looking at isolation housing of social defeat rats found that anxiety-like behaviour was increased in animals housed individually after stress exposure (Nakayasu & Ishii, 2008). The study indicates

the importance of social support on anxiety behaviour of socially defeated mice. This study has been replicated in a social defeat and witness model, in adolescent mice, which found that the witness mice benefit from the support provided by pair housing, whereas the socially defeated mice do not benefit and develop social impairment as a result of the stress (Li et al., 2018). This may be the reason why there was slightly reduced plasma corticosterone release in the witness group and no evident voiding changes in the present study. Social impairment was observed in the present study after stress exposure, when defeated mice, placed back in the cage with their 'buddy' witness mouse, would initiate fighting for a short time. This social ineptitude of defeated mice may be worthy of further investigation.

Effects of Stress on Bladder Physiology

Local bladder mechanisms were investigated to ascertain the changes which contributed to the altered voiding phenotype observed in the social defeat mice. A number of ex-vivo experiments were conducted on isolated whole bladders after euthanasia. Bladder compliance, contractile response and serosal and urothelial mediator release were assessed.

Release of signalling molecules from the bladder urothelium plays an important role in bladder function. Any change in this release has been linked to changes in bladder function (Merrill et al., 2016). Stretch of the bladder has been linked to the release of several mediators including ATP, involved in purinergic sensory stimulation to promote the micturition reflex (Yoshida et al., 2009). While the role of urothelial ATP released has been discussed within the literature in detail, the role of urothelial ACh is still being identified. Several theories involve stimulation of urothelial pacemaker activity and induced release of UDIF from the urothelium to inhibit detrusor muscle contraction

(Hawthorn et al., 2000; Templeman, 2002). Total ACh release into the intraluminal fluid was increased in the social defeat stressed group compared to witness and unstressed group. As stated previously, the role of urothelial ACh is not fully understood, however, there is some evidence that stimulation of muscarinic receptors in sensory nerves depresses sensory transduction (Daly, Chess-Williams, Chapple, & Grundy, 2010). Due to the fact that bladder compliance was unchanged across all groups, there may be a link between the increased urothelial ACh release and decrease urinary frequency seen in the social defeat but not witness group, as a larger urine volume is accommodated before afferent nerves are activated.

Contractile responses to receptor independent (KCl) detrusor stimulation and muscarinic (carbachol) stimulation were not affected by stress. There was some change within the beta-adrenoceptor mediated bladder relaxation, however, this can be linked to the slight increase in the pre-contraction to carbachol. The main change observed with stress was in purinergic stimulated contractions. Stimulation of receptors with αβmATP produced a significantly greater pressure response in bladders from social defeat mice. Similarly, the nerve mediated contractions seen with EFS were increased at all frequencies in the social defeat group. This indicates that social defeat stress increases intravesical pressure generated during voiding contractions which is likely to contribute to the larger void size observed during voiding pattern analysis.

The reason for this enhancement of the purinergic component is not entirely clear. The response to αβmATP indicates an enhancement of the purinergic component of the bladder. Studies have shown that there is co-transmission of ATP and Ach in the bladder, and the purinergic (ATP) and muscarinic (Ach) component play an integral role in the contractility of the mouse detrusor smooth muscle. A study using normal male mice and P2X1-deficient mice found that in the absence of P2X1 receptors, atropine abolished

nearly all nerve-evoked contractions in mice. This demonstrates that contraction of the mouse urinary bladder is dependent on both purinergic and muscarinic signalling (Heppner et al., 2009). This finding is also supported by Burnstock (2013), which indicated that there is equal excitatory co-transmission with purinergic and cholinergic components working via P2X1 and muscarinic receptors respectively. Our study shows that there was no change to the cholinergic responses of the bladder between any of the groups. The addition of atropine had a similar effect on responses to EFS in all groups indicating no change in the cholinergic component. Therefore, with changes in the response to αβmATP and EFS nerve-evoked responses, it appears that social defeat stress causes an upregulation in the purinergic component.

Purinergic signalling within the bladder has been well documented to be involved in a number of physiological and pathophysiological conditions of the lower urinary tract. Studies have shown that purinergic stimulation of P2X7 receptors is involved in inflammation and fibrosis in the bladder (Knight, 2002). Within the human bladder, 3% of neurotransmission is reliant on the purinergic component of stimulation, however, in diseased conditions, the purinergic component is increased to 40% (Burnstock, 2013), particularly in the case of interstitial cystitis and bladder pain syndrome (Birder et al., 2003).

CONCLUSION

The results presented in this chapter indicate that psychological stress, induced by social defeat affects voiding and local bladder function in male mice. The enhanced urothelial ACh release observed following social defeat may desensitize bladder afferent nerves, which coupled with increased nerve evoked voiding contractions may contribute to the decrease in urinary frequency and increased void size, previously referred to as 'retention', of social defeat mice but not witness mice. This change is concluded to be due to enhanced purinergic responses not evident within the witness group. Stress induced bladder changes are dependent on the type of stressor, with no voiding changes observed in the witness trauma model.

CHAPTER 4: EFFECTS OF WATER AVOIDANCE STRESS ON BLADDER FUNCTION AND HOW STRESS-FREE RECOVERY AFFECTS PHYSIOLOGICAL CHANGES

The results presented in Part 1 of this chapter have been published: West, Eliza G., Sellers, Donna J., Chess-Williams, Russ., McDermott, Catherine. (2020). "Bladder overactivity induced by psychological stress in female mice is associated with enhanced bladder contractility", *Life Sciences*, 2020, 118735, https://doi.org/10.1016/j.lfs.2020.118735.

INTRODUCTION

Bladder Pathologies

Healthy human bladder urodynamics are defined by low detrusor pressure and phasic activity during filling and adequate flow and complete emptying during voiding. Several bladder pathologies affect these processes leading to dysfunction.

Overactive bladder (OAB) is defined by the International Continence Society as urinary urgency, with or without urge incontinence and is usually accompanied by frequency and nocturia in the absence of urinary tract infection or other pathologies (Haylen et al., 2010). OAB is known to negatively impact a patient's quality of life and whilst more common in older patients, is also known to affect children and young adults. The most common symptoms of OAB occur during bladder filling where patients experience micturition urgency and some associated incontinence (Arrabal-Polo et al., 2012). Neurogenic and myogenic origins are believed to contribute to OAB. Neurogenic bladder dysfunction often occurs with other neurological diseases and injuries such as spinal cord injury or pelvic and sacral fractures, while myogenic dysfunction refers to changes to the properties of the detrusor muscle (Yoshimura & Chancellor, 2004). In many instances though, the cause of OAB symptoms remains idiopathic (Leron, Weintraub, Mastrolia, & Schwarzman, 2018).

Interstitial cystitis/Painful bladder syndrome (IC/PBS) is a chronic debilitating condition, sharing common symptoms with OAB including urinary urgency and frequency, but, unlike OAB, is also associated with pelvic pain (Marinkovic, Moldwin, Gillen, & Stanton, 2009). The condition is associated with local inflammation of the bladder wall, with an absence of bacterial urinary tract infection (Sant, 2002). Afferent mechanisms are likely to contribute to pain experienced by a patient, however, the aetiology of the symptoms remains unclear.

Psychological stress can impact a number of visceral functions with pathological consequences, and a significant amount of evidence has linked disorders such as OAB and IC/PBS with psychological stress and stress disorders such as anxiety, depression and post-traumatic stress disorder (Lai, Morgan, Vetter, & Andriole, 2016). Lai (2015), performed a case control study to assess the psychological stress levels and perceived stress of OAB and IC/PBS patients and found that stress levels were equally elevated in both patient groups, with a positive correlation between perceived stress and urinary incontinence symptoms in the patients reporting only OAB compared to the controls. The study concluded that there was an overall impact on quality of life, which in turn can further exacerbate symptoms (Lai, 2015). It has also been reported that childhood sexual trauma was more prevalent in patients with OAB (29.4%) compared to controls (6.7%), with this group reporting more pain symptoms and poorer mood, however, this did not correlate with the severity of OAB symptoms (Lai et al., 2016). A cohort study investigated the incidence and remission of OAB in female US veterans and identified the impact of the symptoms on overall mental health. The study found that anxiety, depression and prior sexual assault influence the natural progression of OAB and recommended that medical providers should screen for mental health conditions with diagnosed OAB (Bradley, 2017).

Environmental Stress and the Water Avoidance Model

While clinical studies show the correlation between psychological stress and exacerbated bladder dysfunction, experimental models of psychological stress have shown that stress may play a causal role in the development of bladder dysfunction (Merrill, Malley, & Vizzard, 2013).

Environmental stress refers to a subjective psychological response to changes in the normal physical environment. The detrimental effects of chronic stress depend on how well the individual adapts to cumulative environmental stress (Clark, Bond, & Hecker, 2007). For humans, environmental stressors include noise, poor housing quality, and crowding amongst others, while in rodents this includes any change to normal housing. This is the basis of the water avoidance stress model, a well-established model of psychological stress that imitates the experience of negative environmental life changes in humans (Lee et al., 2016). If stress is prolonged, it can have adverse effects on several physiological functions (Herman, 2013). As stated previously, the exposure to physical and emotional stress produces a hormonal stress response, which has been measured in a number of rodent models of psychological stress. The water avoidance stress model has previously been used to assess the development of bladder dysfunction, with 6-week-old male mice developing moderate bladder wall hypertrophy and increased voided volumes (McGonagle et al., 2012). However, water avoidance stress in female Wistar rats is reported to increase micturition frequency and urinary noradrenaline, in addition to reducing mechanical pain threshold causing hyperalgesia (Matos, 2017). Similarly, Smith et al (2011) found that female rats developed increased micturition frequency and decreased latency to void following acute and chronic water avoidance stress (Smith et al., 2011); this was associated with increased vascularity, in addition to increased total and activated mast cells in the bladder. Due to the overlap in brain circuits involved in

stress, anxiety and micturition, another study by Wang et al. (2017), evaluated the effects of chronic stress on the brain during bladder filling. The study found that there was a greater activation of the micturition centre in the stressed animals, which lead to increased urgency to void during bladder filling, contributing to both an OAB and IC/PBS phenotype (Wang et al., 2017). Another study has looked more closely at the role of inflammation and its impact on bladder function, in the water avoidance stress response. The study found that chronic stress, induced by perpetual water avoidance stress, caused urinary frequency, and linked this symptom to cyclooxygenase-2 gene upregulation in the bladder smooth muscle cells (Yamamoto et al., 2012). These studies have shown that psychological stress plays a causal role in the development of bladder dysfunction, however, the local bladder mechanisms causing these changes remains generally unknown.

Recovery from Psychological Stress

Recovery of bladder pathologies after stress has not been well documented. However, due to the recurring nature of psychological stress it is an important aspect that must be considered when assessing physiological change. Very few animal models of psychological stress have assessed the effects of recovery on physiological functions altered with stress. The water avoidance stress model has been used by one group to assess recovery of voiding frequency. The study reported that water avoidance stress increases micturition frequency and that these voiding changes persisted for 1 month, however, no voiding data was shown in the published paper (Smith et al., 2011). Another study has observed the effects of water avoidance stress on visceral and somatic nociception, colonic motility, and anxiety-like behaviour. The study found that chronic water avoidance stress of rats resulted in long lasting, persistent visceral hyperalgesia

(Bradesi et al., 2005). A male mouse social defeat model was used over a 30-day period to stimulate psychoemotional stress which led to anxious and depressive symptoms. After psychological stress ceased, mice were housed for 2 weeks in comfortable conditions with other male mice. The study found that even after cessation of psychological stress, the male mice continued to show symptoms of anxiety and depression in behavioural tests (Avgustinovich, Kovalenko, & Kudryavtseva, 2005). Clinical examination of cortisol responses to psychological stress was assessed in a separate study which found that non-depressed and clinically depressed individuals exhibited similar baseline cortisol levels. In the recovery period however, clinically depressed patients had a significantly higher cortisol level compared to their non-depressed counterparts (Burke, Davis, Otte, & Mohr, 2005). Several clinical studies have also observed that delayed recovery from psychological stress leads to poor cardiovascular outcomes and may predict earlier death (Chatkoff, Maier, & Klein, 2010). It is clear from the few studies mentioned above that chronic psychological stress results in persistence of pathological symptoms. Besides the single study mentioned, the long-term effect of psychological stress on bladder function has not been investigated. However, with the water avoidance recovery model, described in this chapter, these bladder changes are able to be assessed.

Aims

The aim of the present study was to investigate the effects of water avoidance stress on murine bladder function. Specific aims were:

- To investigate the effects of water avoidance stress on voiding behaviour.
- To determine the effects of water avoidance stress on local bladder function. including bladder compliance, contractile responses and urothelial mediator release.
- To assess how a stress-free recovery period impacts voiding behaviour and local bladder mechanisms induced by water avoidance stress.

METHODS

Animals

Young adult female C57BL/6J mice were obtained and housed as outlined in the general methodology chapter (Chapter 2). Mice were randomly allocated to the following groups: Study 1: Control (Unstressed) or Water Avoidance Stress (Stressed) and Study 2: Control (Unstressed), Water Avoidance Stress (Stressed) or Recovered (Stress + 10-days). Animal cohorts in Part 1 and Part 2 were completely separate.

Water Avoidance Stress Protocol

Adult female (12-14 weeks) mice were used for Water Avoidance Stress (WAS) studies. The WAS protocol was adapted from several previous stress protocols (Bradesi et al., 2005; McGonagle et al., 2012; Wang et al., 2017). The WAS chamber was custom made, measuring 30 cm in diameter and 40 cm high. Within this larger chamber, a smaller podium was fitted (7.5 cm in diameter and 20 cm high). The height of the larger tube was to ensure the mice could not jump out during the 1-hour stress protocol. A diagram of this structure is shown below **(Figure 4.1A)**.

Distilled water was used to fill the chamber to 2 cm below the central podium. A photograph is shown below of mice surrounded by distilled water within the chamber **(Figure 4.1.B)**. This protocol was consistent with other water avoidance stress protocols found in the literature.

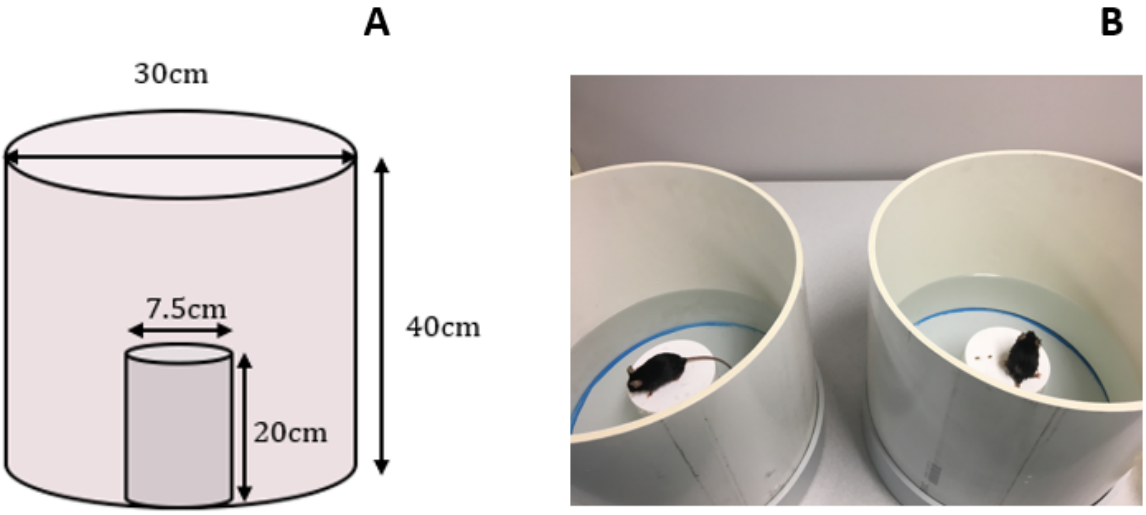

Figure 4.1: (A) Diagram displaying the dimensions of the water avoidance chamber (Created by the author) and (B) C57BL/6J mice sitting on the podium during the water avoidance stress protocol (Photograph taken by the author).

Mice were removed from their normal housing and placed onto the podium surrounded by water for 1 hour. During this time, the mice were observed in case they fell off the podium. In most cases, the mice were able to climb back onto the podium if they fell off, and if not, the mice were placed back onto the podium. After 1 hour, mice were placed back into their cage, the water was emptied, and the chamber was cleaned with 80% ethanol solution. Mice in the stress group were placed in the chamber for 1 hour per day for 10 consecutive days. Control (unstressed) mice were age-matched and housed under normal conditions without environmental stress or movement from normal housing.

Recovery Protocol

Study 2 of this chapter investigates the potential for recovery from the impacts of psychological stress by allowing mice who have experienced 10-days WAS, a 10-day stress-free period. Animals were age matched and underwent the same stress exposure as mice in the original stress group in study 1, however instead of being sacrificed on day 10, they were maintained in normal housing to recover without further stress for 10-days. Voiding pattern analysis for unstressed and stressed animals occurred as described in chapter 2, undertaken on days 0, 1, 3, 5, 7 and 10. For animals that underwent the stress-free recovery, VPA continued on days 11, 13, 15, 17 and 20. Age matched controls also underwent VPA on these days. These animals were then sacrificed on day 20 following a final VPA and whole bladder preparations were undertaken as described below.

Whole Bladder Preparation

Whole bladder preparations were performed as described in chapter 2, with the following differences due to the sex of mice. When the abdominal region was secured to the dissection bath under the microscope, female reproductive organs were removed including, uterus, fallopian tubes, ovaries and surrounding connective tissue. The whole bladder preparation then continued as described in chapter 2.

Statistical Analysis

Two-way ANOVA with Tukey's post hoc test for multiple comparisons was used to compare the different time point and voiding variables. The same statistical tests were also used to analyse the concentration-response curves. Student's t-test was used to compare the difference between the two animal groups (Urothelial mediator release data, animal parameters, spontaneous activity data, response to ATP and KCl) in Part 1. One-way ANOVA with Bonferroni post-hoc test was used to compare the difference between the three animal groups (Urothelial mediator release data, animal parameters, spontaneous activity data, response to ATP and KCl) in Part 2.

RESULTS

PART 1: Impact of WAS on bladder function

Animal Parameters and Voiding Behaviour

Animal body weight and water consumption were measured on day 0 to obtain baseline data. The body weight and water consumption of mice was also then measured on days 1, 3, 5, 7 and 10. This data was not altered by WAS compared to the unstressed control **(TABLE 4.1)**. Bladder weight was measured at the end of the whole bladder preparation and was also unchanged in the water avoidance stress group compared to the unstressed control.

TABLE 4.1: *Baseline body weight, water consumption and bladder weight (at day 10) in control (unstressed) and water avoidance stress mice (stressed) (n=6).*

	Unstressed	**Stressed**
Body weight (g)	18.20 ± 0.48	18.50 ± 0.67
Bladder weight (mg)	21.70 ± 1.08	20.78 ± 0.53
Water consumption (g)	0.67 ± 0.21	0.79 ± 0.23

At the time of euthanasia, a blood sample was taken for plasma corticosterone analysis. **Figure 4.2** shows a significant increase in plasma corticosterone level in the water avoidance stress group (stressed), 154.20 ± 29.24 μg/mL (n=6), compared to the unstressed controls, 54.14 ± 9.14 μg/mL (n=6).

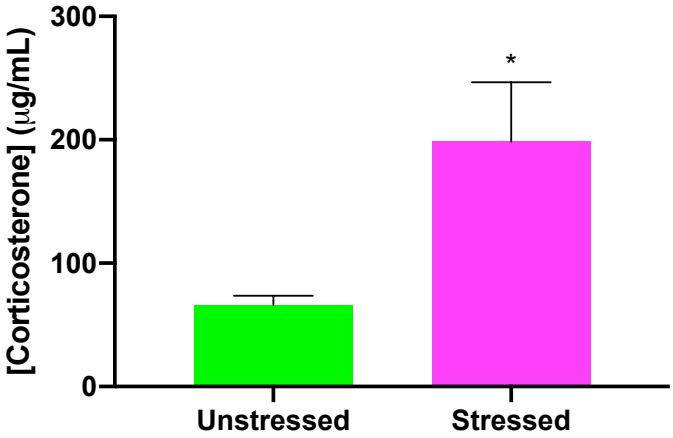

Figure 4.2: Concentration of plasma corticosterone in in Stressed and Unstressed mice. Datum is represented as mean ± SEM (n = 6). Analysis was performed by an unpaired Student's t-test (*$p < 0.05$).

Voiding pattern analysis was undertaken to examine voiding behaviour, including number of voiding events, average voided area, total voided area and number of voids smaller than 0.2 cm^2. There was no significant difference in the total voided area **(Figure 4.3.C)** in the stressed animals compared to the unstressed animals, indicating that urine production was not affected by water avoidance stress. There was, however, an increase in urinary frequency in the stressed group compared to the unstressed group **(Figure 3.4.A)**. This increase was significant at day 3 ($p = 0.013$), day 5 ($p = 0.005$), day 7 and day 10 (both $p = < 0.001$). Although variable across the 10-days of stress exposure, average void size **(Figure 4.3.B)** decreased in the stressed group compared to the unstressed controls, with a statistically significant reductions at day 3 ($p = 0.029$) and day 6 ($p = 0.014$). This fits with **Figure 4.3.A**, with the number of voids increasing while the average void size decreases, meaning that the stressed animals were urinating smaller amounts, less frequently. This change is also reflected in **Figure 4.3.D** where the number of small voids below 0.2cm^2 increases over time, and significantly increased at day 3 ($p = 0.005$), day 5 ($p = 0.001$), day 7 ($p < 0.001$) and day 10 ($p < 0.001$). The

absence of change in both water consumption **(Table 4.1)** and total voided volume indicate that the change observed in the stressed group is an actual change in urinary frequency and not increased urine production.

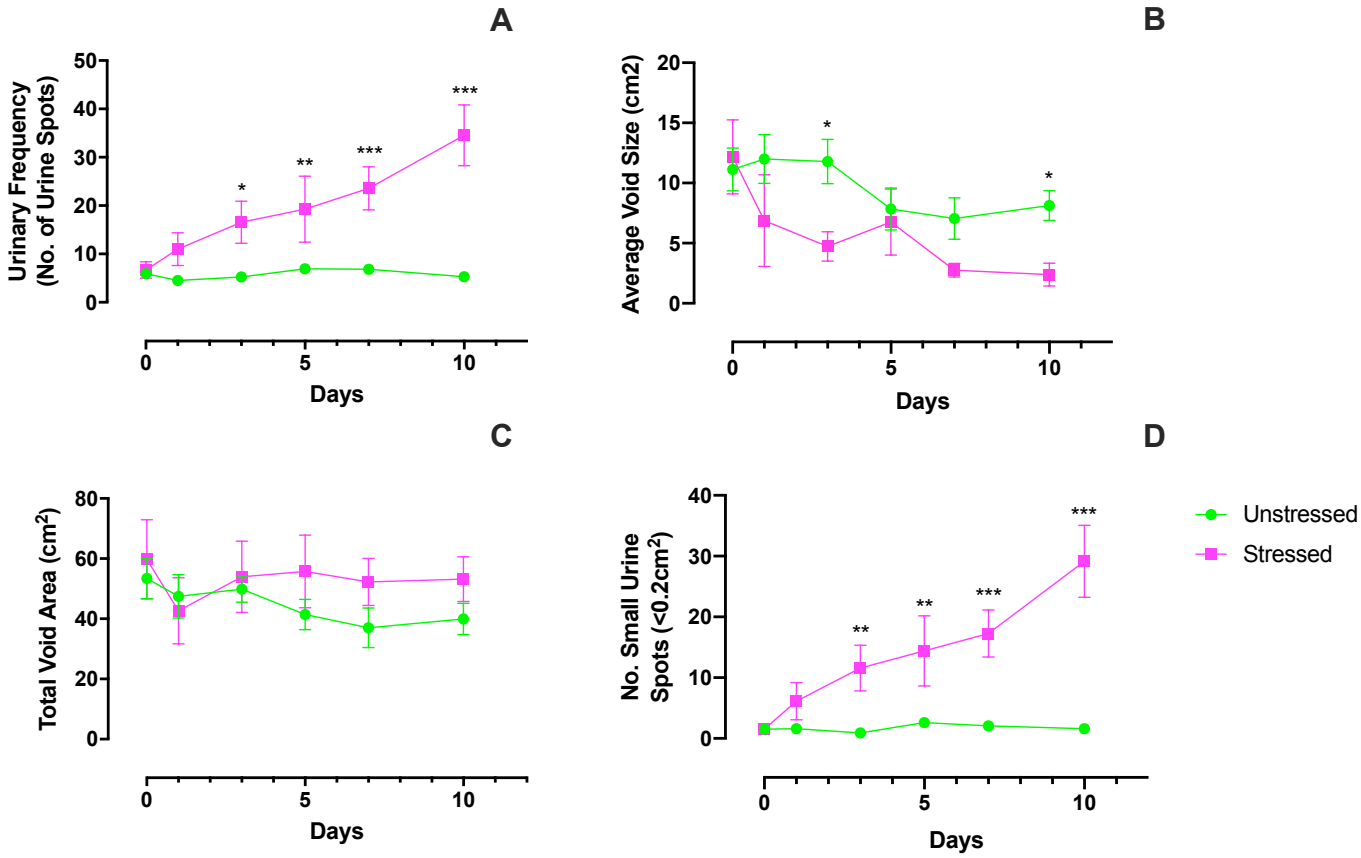

Figure 4.3: Voiding pattern analysis conducted in Stressed and Unstressed mice. (A) Number of voiding events, (B) Average voided area, (C) Total voided area and (D) Number of small urine voids smaller than 0.2 cm². Datum is presented as mean ± SEM (n = 7). Analysis was performed using two-way repeated measures ANOVA with Bonferroni multiple comparisons test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Unstressed vs. stressed)

As stated previously, at the time of filter paper collection after voiding, faecal pellets were collected from the voiding paper and left to dry overnight. The pellets were then counted and weighed the next day. Faecal pellet data was variable across all days, with no significant difference between the stressed and unstressed control animals **(Figure 4.4)**.

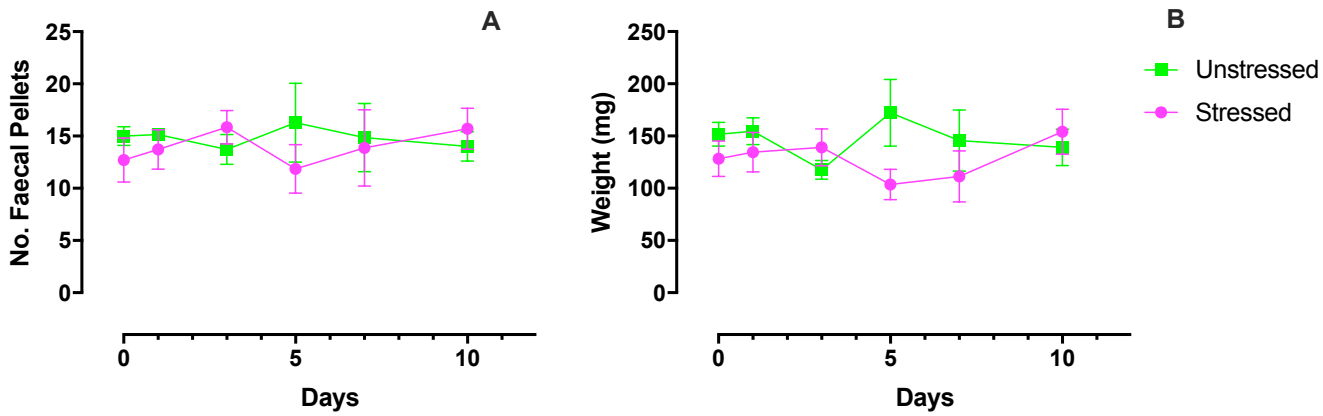

Figure 4.4: Faecal pellet analysis conducted in in Stressed and Unstressed mice. (A) Number of faecal pellets, (B) relative weight of faecal pellets. Datum is presented as mean ± SEM (n = 7). Analysis was performed using two-way repeated measures ANOVA with Bonferroni multiple comparisons test.

Mediator Release

The intraluminal fluid, taken from distended bladders (20 mmHg intraluminal pressure), and the serosal fluid, taken from the water bath, were analysed for ATP and Ach content. The concentration of each mediator in individual samples was quantified and total amount released calculated to account for intraluminal and serosal fluid volume. Both luminal ATP and ACh concentrations were greater compared to the serosal fluid. Water avoidance stress did not affect the total amount of ATP or Ach release from the lumen or serosa of the mouse bladders **(Figure 4.5)**.

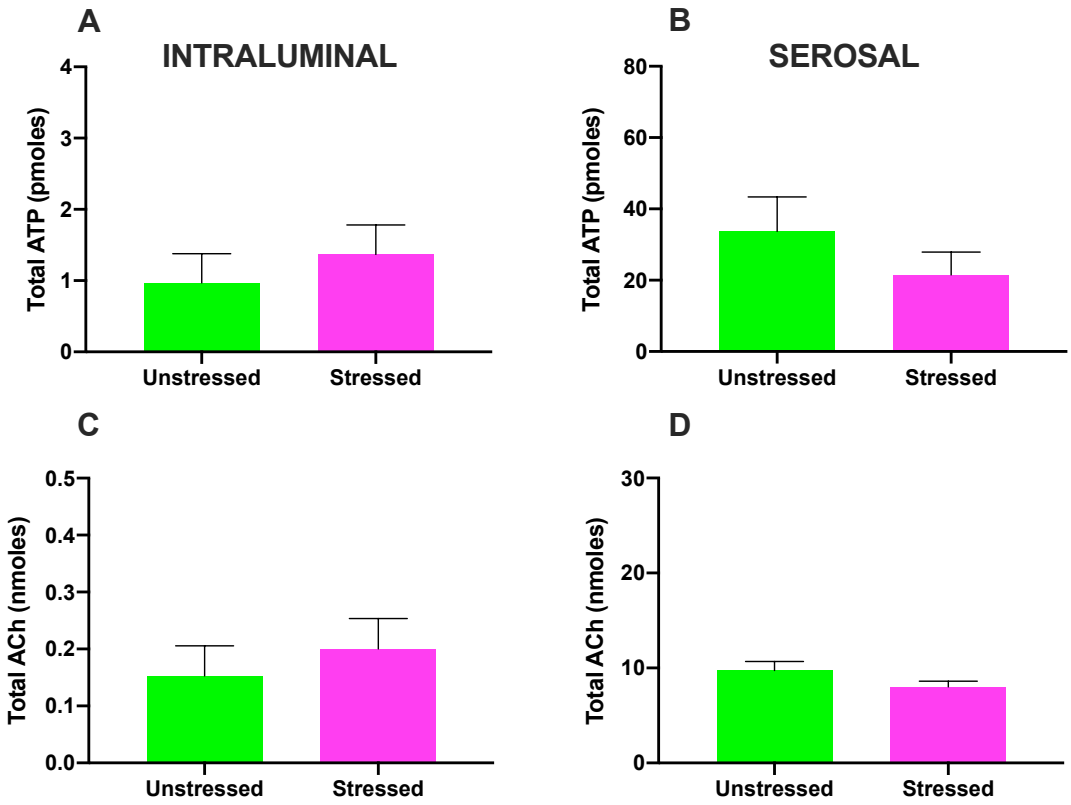

Figure 4.5: Total release of ATP and ACh into the (A & C) intraluminal and (B & D) serosal fluid collected following distensions of isolated bladders from Stressed and Unstressed mice. Datum is represented as mean ± SEM (n = 6). Analysis was performed by an unpaired Student's t-test.

Bladder Compliance and Stretch-relaxation

Bladders were filled with Krebs-bicarbonate solution to a physiological pressure of 20 mmHg. A volume-pressure relationship was used to measure bladder compliance across the Unstressed and Stressed groups. Bladder compliance was not significantly different in the stressed compared to the unstressed group **(Figure 4.6)**.

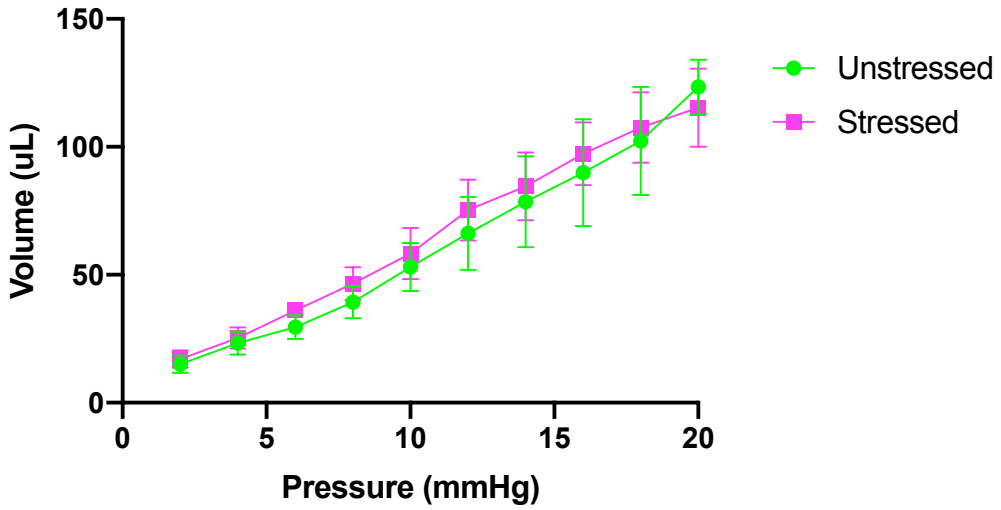

Figure 4.6: Volume pressure relationship for bladders from in Stressed and Unstressed mice. Datum is represented as mean ± SEM (n = 6), analysed by two-way ANOVA.

After fluid collection was completed, bladders were infused with Krebs-bicarbonate solution to a pressure of 20 mmHg from baseline, at which point the perfusion pump was switched off and the outflow tap remained closed. The bladders were left to accommodate the pressure and stabilise for 30 minutes to equilibrate. Over the course of 30 minutes, the bladders relaxed and pressure plateaued. The pressure-time relationship was then plotted to observe overall stretch-relaxation of the equilibration period. There was a sharp decrease in the initial 5 minutes of relaxation after stretch, therefore, the 'X axis' of the graph is split and extended to highlight this change. There was no significant difference between the Unstressed and Stressed groups **(Figure 4.7)**.

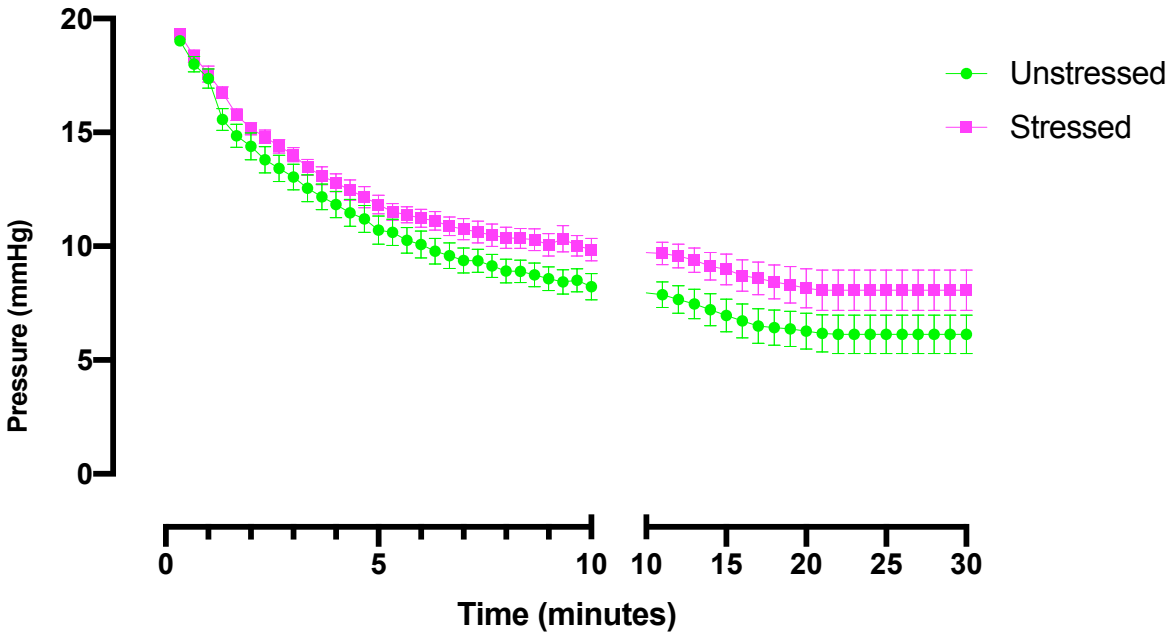

Figure 4.7: Pressure time relationship for bladders from Stressed and Unstressed mice. Datum is represented as mean ± SEM (n = 6), analysed by two-way ANOVA.

Bladder Contractility and Response to Electrical Field Stimulation

Bladder contractile response to KCl was measured with data recorded as a change in pressure from baseline, to assess the impact of WAS on receptor-independent, general contractility of the bladder **(Figure 4.8)**. There was a significant increase in the response to KCl in bladders from stressed mice compared to unstressed controls with pressures recorded (25.90 ± 2.77 mmHg and 17.1 ± 1.91 mmHg respectively, n=6) ($p = 0.03$).

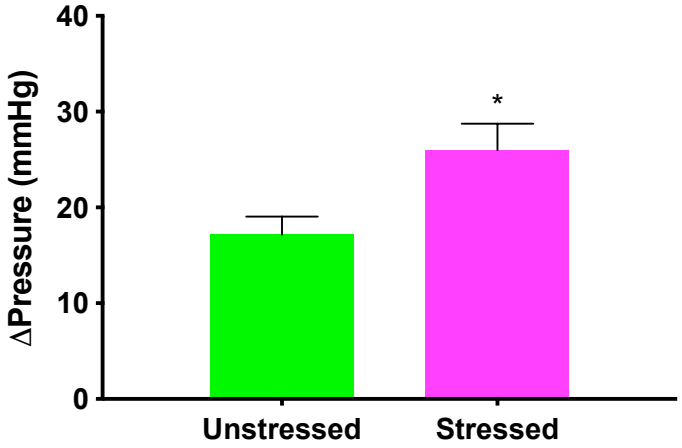

Figure 4.8: Pressure responses to KCl (60 mM) in Stressed and Unstressed mice. Datum is represented as mean ± SEM (n = 6) and analysed using an unpaired t-test (*$p < 0.05$).

To investigate the impact of WAS on nerve evoked contractile responses, bladders were electrically stimulated at 1, 5, 10 and 20 Hz. Stress did not change responses to EFS **(Figure 4.9.A)**, and when this response was normalised to the KCl response the nerve mediated contractions was not significantly different between groups **(Figure 4.9.B)**.

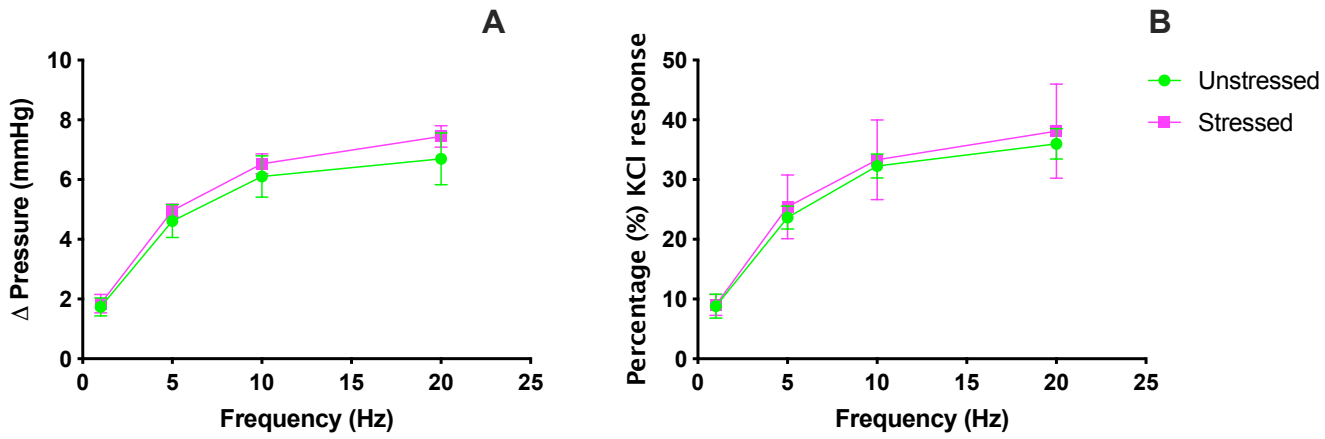

Figure 4.9: Nerve evoked pressure response of isolated bladders from Stressed and Unstressed mice at 1, 5, 10 and 20 Hz. Responses were recorded as (A) a change in pressure from baseline and (B) a percentage of the KCl contractile response. Datum is presented as the mean ± SEM (n = 6) and analysed using a two-way repeated measures ANOVA.

Addition of the NOS inhibitor LNNA did not significantly alter bladder contraction to EFS in either of the groups, indicating the inhibitory neurotransmitter, NO, was not involved in neurotransmission **(Figure 4.10.A)**. Addition of the muscarinic antagonist, atropine (1 μM), reduced the response to EFS (42.50 ± 4.87%, n=6) in unstressed bladders, this change was less in the stressed group (28.90 ± 4.12%, n=6) **(Figure 4.10.A and B)**. Desensitisation of purinergic receptors with $\alpha\beta$mATP (1 mM) reduced the responses to EFS to a similar extent in bladders from both groups (Unstressed 50.50 ± 4.50%, Stressed 65.69 ± 4.44%, n=6) with a significant difference detected between the groups ($p = 0.04$) **(Figure 4.10.A and C)**. This demonstrates that while nerve contractions were not changed by water avoidance stress, the relative contribution of ACh was decreased while the ATP contribution was slightly increased.

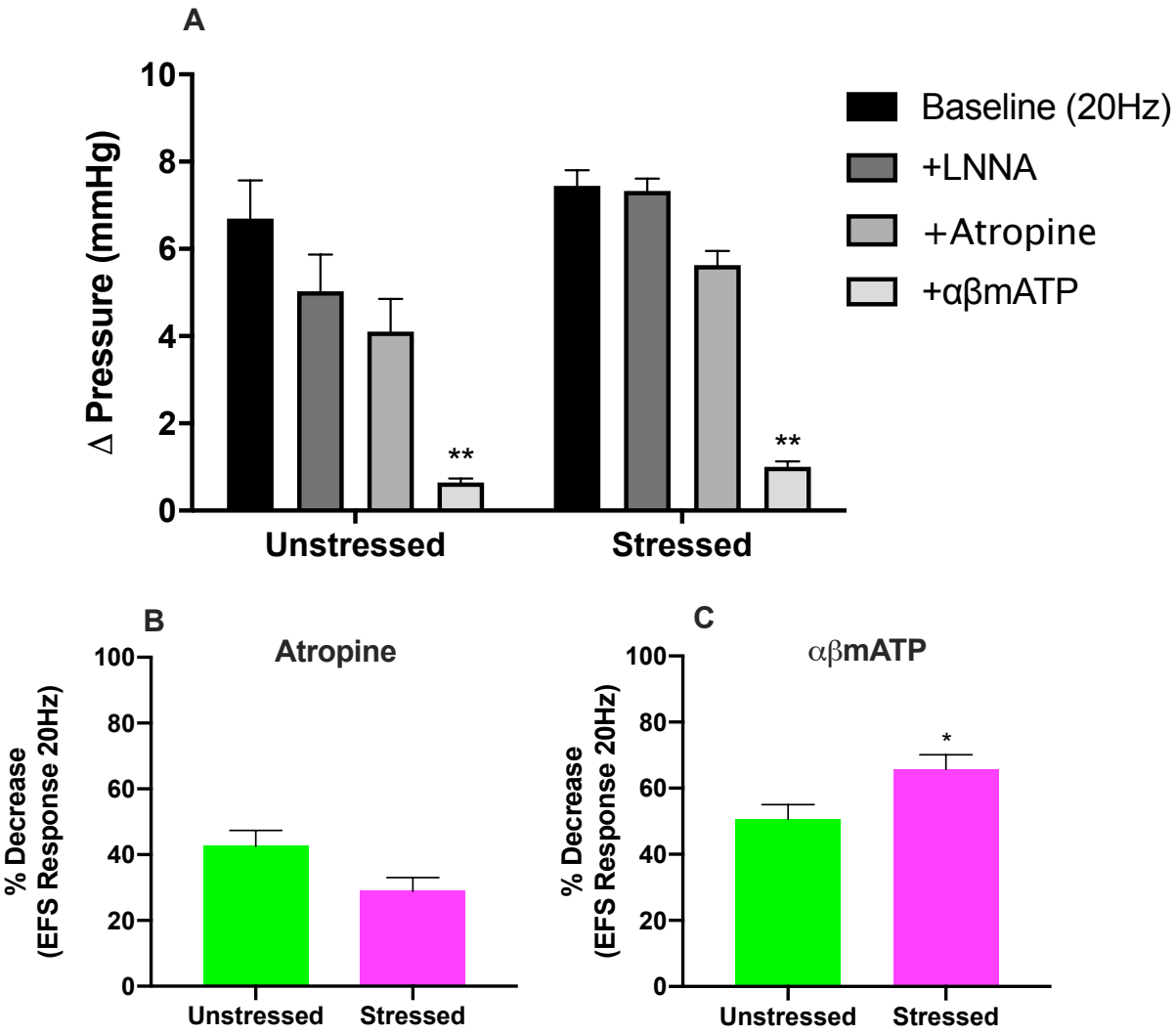

Figure 4.10: (A) Intravesical pressure responses to electrical field stimulation at 20 Hz (baseline), and after addition of L-NNA (100 μM), atropine (1 μM) and αβmATP (1 mM) to bladders from Stressed and Unstressed mice bladders. Percentage decrease in EFS response on addition of (B) atropine and (C) αβmATP to bladder from each group. Datum is expressed as mean ± SEM and analysed using two-way repeated measures ANOVA with Dunnett's multiple comparisons test for (A) and one-way ANOVA with Dunnett's multiple comparisons test for (B and C) (*$p < 0.05$, **$p < 0.01$ vs. Baseline (20 Hz)).

Response to Pharmacological Agents

Pharmacological agents were added to the bath and pressure responses were recorded as a change from baseline. Purinergic stimulation was assessed using ATP (10 mM) and $\alpha\beta$mATP (1 mM). The response to ATP **(Figure 4.11.A)** was significantly increased in the Stressed group compared to the Unstressed group with change in pressure from baseline (2.43 ± 0.58 mmHg and 1.37 ± 0.01 mmHg ($p = 0.0106$), respectively, n=6). The contractile responses were also normalised to KCl response resulting in no significant difference between groups **(Figure 4.11.B)**.

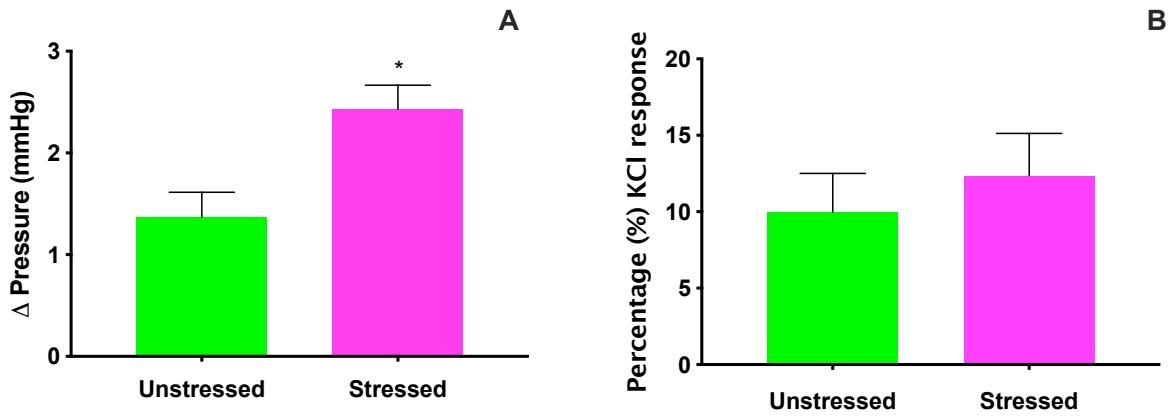

Figure 4.11: Contractile responses to the purinergic agonist (A) ATP (10 mM) and (B) ATP response normalised to the KCl response in Stressed and Unstressed mice bladders. Datum is represented as mean ± SEM (n = 6) and analysed using an unpaired Student's t-test (*$p < 0.05$).

After P_2X receptor desensitisation with αβmATP the initial contraction was recorded **(Figure 4.12)**. Compared to the Unstressed bladders (0.65 ± 0.09 mmHg, n=6) there was a significant increase in contractile response in bladders from the stressed group (1.00 ± 0.31 mmHg, n=6) ($p = 0.043$). The contractile responses were also normalised to the KCl response resulting in no significant difference between groups **(Figure 4.12.B)**.

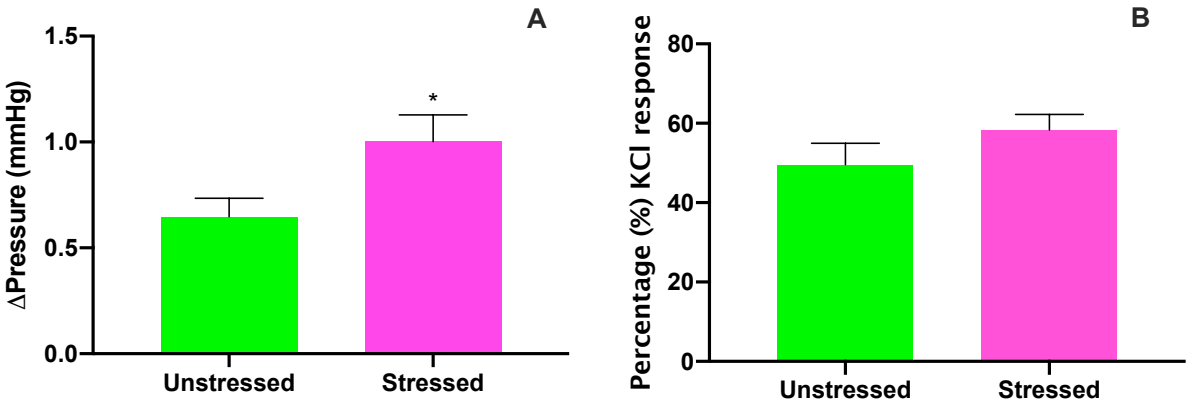

Figure 4.12: Pressure responses to $\alpha\beta$mATP (1 mM) in in Stressed and Unstressed mice bladders. Datum is represented as mean ± SEM (n = 6) and analysed using an unpaired t-test (*$p < 0.05$).

A cumulative concentration-response to carbachol (muscarinic agonist) was performed on each bladder to study changes in the muscarinic-receptor-mediated response **(Figure 4.13)**. Analysis was performed by a two-way ANOVA with Tukey multiple comparisons test and showed a significant increase in the maximum contraction in bladders from the stressed group compared to the unstressed group **(Figure 4.13.A)**, with no significant difference in pEC_{50} **(Table 4.2)**. The concentration-response curve was also normalised as a percentage of the KCl response, resulting in no significant difference between the groups **(Figure 4.13.B)**.

TABLE 4.2: *Whole bladder responses to carbachol in control (unstressed) and stressed mice (n=6).*

	Unstressed	**Stressed**
pEC_{50}	5.42 ± 0.11	5.41 ± 0.07
Maximal response		
ΔPressure (mmHg)	23.38 ± 3.39	30.87 ± 2.48 ($p < 0.01$)
Response (% of KCl)	115.99 ± 7.67	124.47 ± 4.91

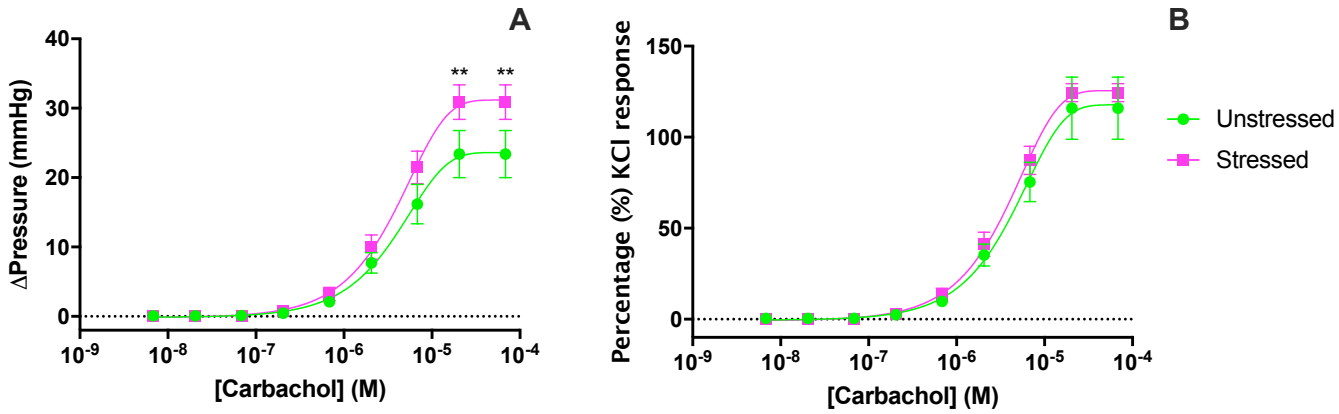

Figure 4.13: Carbachol concentration-response curves, in Stressed and Unstressed mice. Responses to carbachol are presented as (A) change in intravesical pressure from baseline and (B) change in intravesical pressure normalised to the KCl response. Datum is represented as mean ± SEM (n = 6), analysed using non-linear regression and two-way ANOVA with Tukeys multiple comparisons test ($p < 0.01$).**

Carbachol (1 μM) was used to pre-contract bladders before relaxation responses to isoprenaline, as a concentration-responses were measured. Maximal relaxation to isoprenaline was significantly greater ($p = 0.008$) in bladders from the stressed group with no change in potency (pIC_{50}) **(Table 4.3)**.

TABLE 4.3: *Whole bladder responses to isoprenaline in control (unstressed) and stressed mice (n=6).*

	Unstressed	Stressed
pIC_{50}	6.87 ± 0.12	6.71 ± 0.16
Maximal response		
Δ Pressure (mmHg)	-3.27 ± 0.50	-4.58 ± 0.25 ($p < 0.01$)
Response (% Decrease)	-18.03 ± 15.04	-30.26 ± 19.96

While maximal relaxation responses were greater ($p < 0.01$) in the stressed group compared to the Unstressed group, there was no significant difference between the groups when relaxation was normalised to the magnitude of carbachol precontraction **(Figure 4.14.B)**.

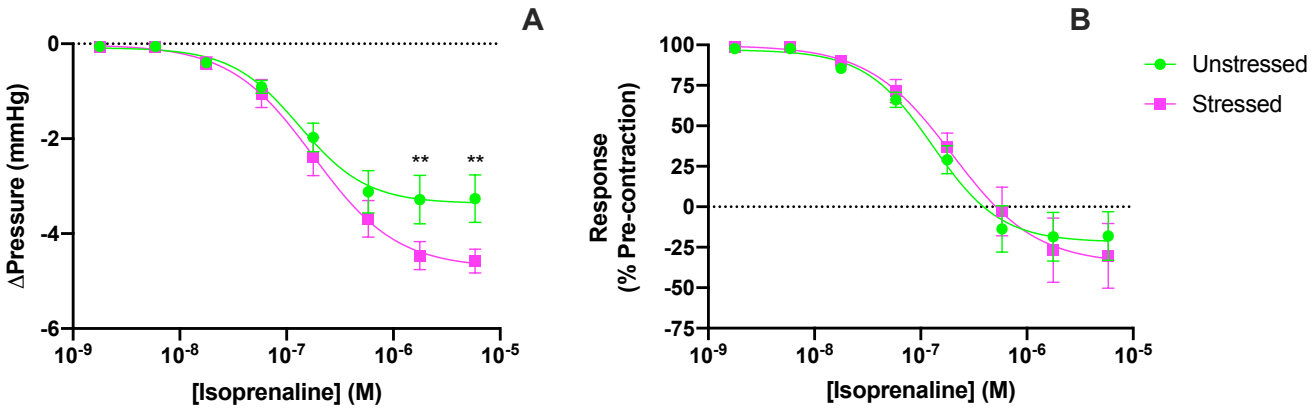

Figure 4.14: Effect of isoprenaline on isolated bladders from Stressed and Unstressed mice with datum given as (A) raw data as change in pressure from pre-contraction, (B) as a percentage (%) of the carbachol precontraction. Datum represented as mean ± SEM (n = 6) and analysed using two-way ANOVA.

Spontaneous Phasic Contractions

Spontaneous activity was measured during bladder stretch-relaxation and the average frequency **(Figure 4.15.A)** and the amplitude **(Figure 4.15.B)** of spontaneous contractile activity was unchanged in bladders from the stressed group compared to the Unstressed.

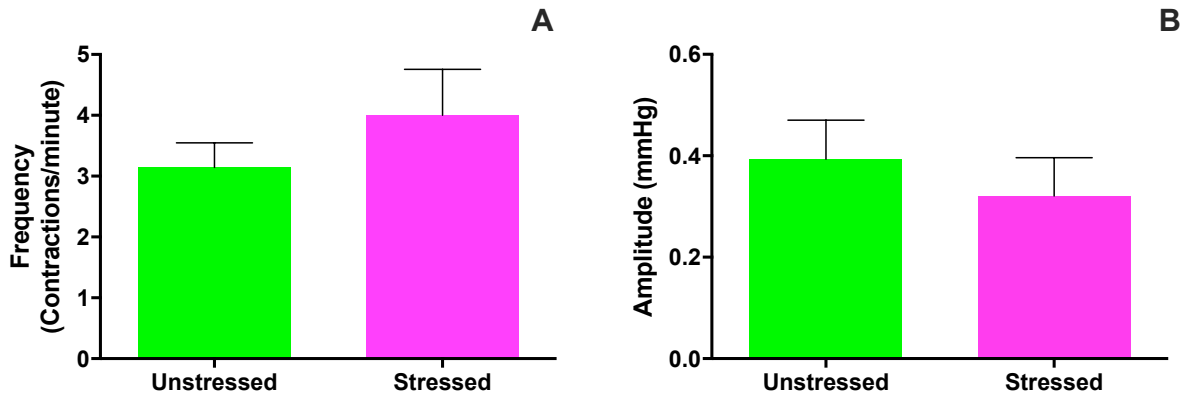

Figure 4.15: Spontaneous phasic contractions in isolated Stressed and Unstressed mouse bladders during stretch-relaxation period. (A) Frequency of contractions and (B) amplitude of spontaneous contractions. Datum is represented as mean ± SEM (n = 6), analysed using unpaired Student's t-test.

Phasic activity was also measured after the addition of 1 μM carbachol to pre-contraction. During this period the phasic contractions following plateau of the tonic contraction were measured showing a significant increase ($p = <0.001$) in the frequency in the stressed group (6.57 ± 0.20 mmHg, n=6) compared to the unstressed group (2.57 ± 0.37 mmHg, n=6) **(Figure 4.16.A)**. Amplitude of phasic activity was also increased significantly ($p = 0.02$) in the stressed group (0.29 ± 0.02 mmHg, n=6) compared to the unstressed control group (0.20 ± 0.02 mmHg, n=6) **(Figure 4.16.B)**.

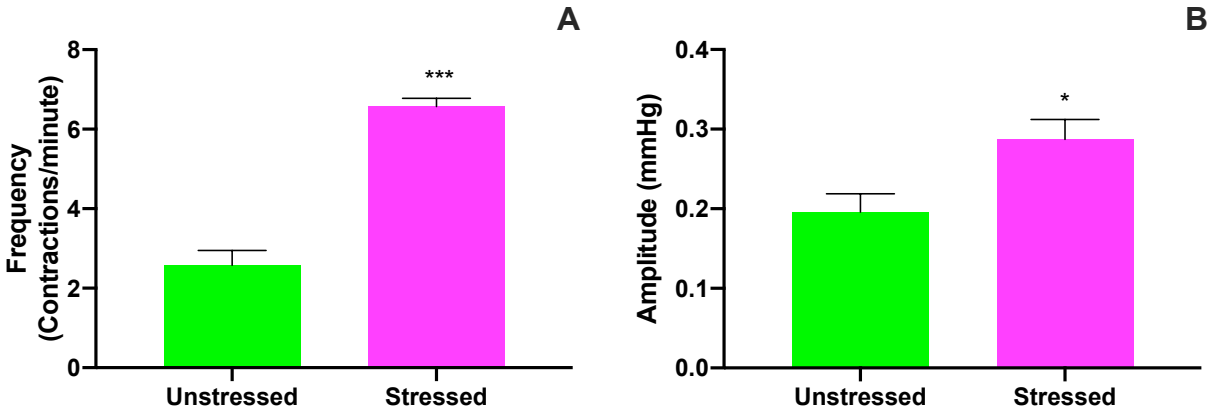

Figure 4.16: Phasic response to tonic contraction of 1 μM carbachol in Stressed and Unstressed mice bladder. (A) Frequency of phasic contractions and (C) Amplitude of phasic contractions. Datum is represented as mean ± SEM (n = 6), analysed using un-paired Student's t-test (*$p < 0.05$, ***$p < 0.001$).

PART 2: Impact of a stress-free period following stress exposure on bladder function

Part 2 of the results will include a stressed group which underwent 10-days of recovery after the 10-days of water avoidance stress. Groups will be as follows; Control (Unstressed), Water Avoidance Stress (Stressed) or Recovered (Stress + 10-days).

Animal Parameters and Voiding Behaviour

Body weight and water consumption was unchanged in the recovered (Stress + 10-days) mice compared to the unstressed controls and stressed mice **(Table 4.4)**. Bladder weight was measured following whole bladder preparation with no significant difference observed between the groups.

TABLE 4.4 *Baseline body weight, water consumption and bladder weight (at day 10 and 20) in control (unstressed), water avoidance stress (Stressed) and recovered (stress + 10-days) mice.*

	Unstressed	Stressed	Stress + 10-days
Body weight (g)	21.2 ± 0.28	18.5 ± 0.67	20.6 ± 0.67
Bladder weight (mg)	23.1 ± 1.13	20.78 ± 0.53	22.3 ± 1.43
Water consumption (g)	0.95 ± 0.06	0.79 ± 0.23	1.15 ± 0.12

Plasma corticosterone levels were significantly increased in the stressed group (154.20 ± 29.24 µg/mL ($p = 0.0048$)) compared to unstressed group (54.14 ± 9.14 µg/mL), with corticosterone levels significantly decreased and returned to unstressed levels after 10-days of stress-free recovery (32.06 ± 0.10 µg/mL) ($p = 0.0031$) **(Figure 4.17)**.

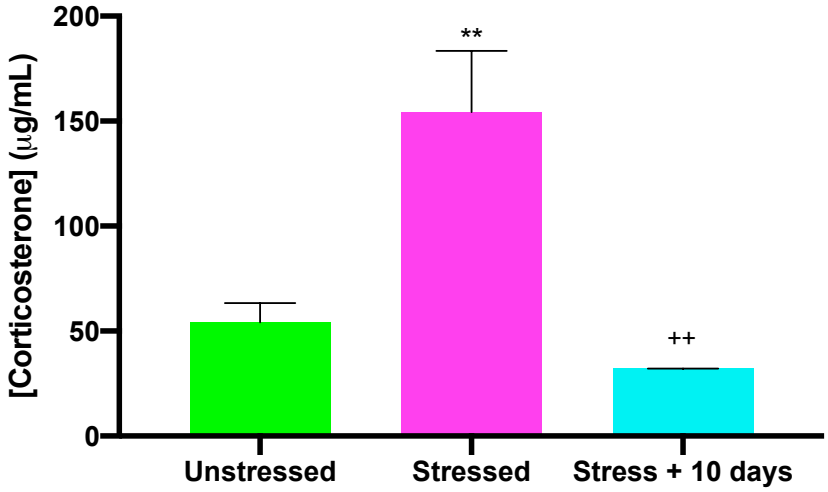

Figure 4.17: Plasma corticosterone levels in Stressed, Unstressed and Stress + 10-days groups. Datum is represented as mean ± SEM (n = 6). Analysis was performed using a one-way ANOVA with Tukey multiple comparisons test ($p < 0.01$, unstressed vs. stressed) ($^{++}p < 0.01$, stressed vs. stress + 10-days).**

Voiding pattern analysis was performed in stressed mice on days 0, 1, 3, 5, 7 and 10 of the stress exposure protocol and continued during the stress-free period on days 11, 13, 17 and 20 to assess recovery of bladder function after stress. Unstressed mice also underwent voiding pattern analysis at the same timepoints, for comparison. Overall, voiding frequency increased in stressed mice over the initial 10-day stress period compared to the unstressed group. Over the following 10-days of stress-free recovery, the voiding frequency decreased although not completely to baseline, remaining significantly elevated ($p = <0.001$) compared to the unstressed group **(Figure 4.18.A)**. Total voided area was unchanged over the 10-days of stress and was similarly unchanged during the stress-free recovery period **(Figure 4.18.B)**. Average void size was reduced in stressed mice over the stress and stress-free recovery period **(Figure 4.18.C)**. The number of small voids was also increased over the 10-days of stress and was still increased in the following 10-day stress-free period ($p = <0.001$) **(Figure 4.18.D)**. These

changes support the voiding behaviour changes reported in Part 1 for the WAS model and suggest that voiding dysfunction does not fully recover in stressed mice following 10-days stress-free.

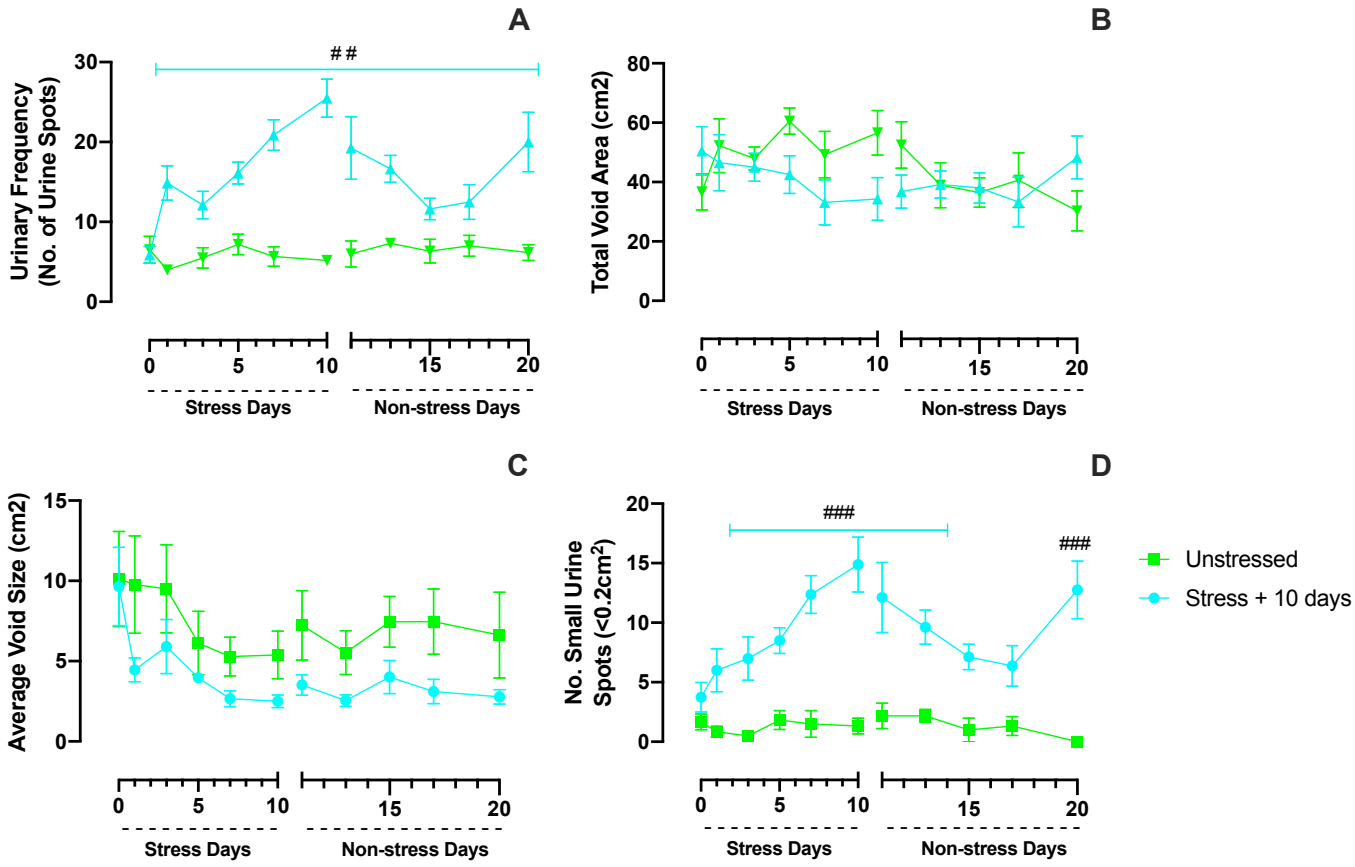

Figure 4.18: Voiding pattern analysis conducted in Stress + 10-days and Unstressed mice. (A) Number of voiding events, (B) Total voided area, (C) Average void size and (D) Number of small voids below 0.2 cm². Datum is presented as mean ± SEM (n = 8). Analysis was performed using two-way repeated measures ANOVA ($^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$).

Faecal pellet data was also collected over the 20 days of stress and stress-free recovery. There was no significant difference in both the number of pellets **(Figure 4.19.A)** and weight of pellets **(Figure 4.19.B)** for the Unstressed versus the stress + 10-days groups.

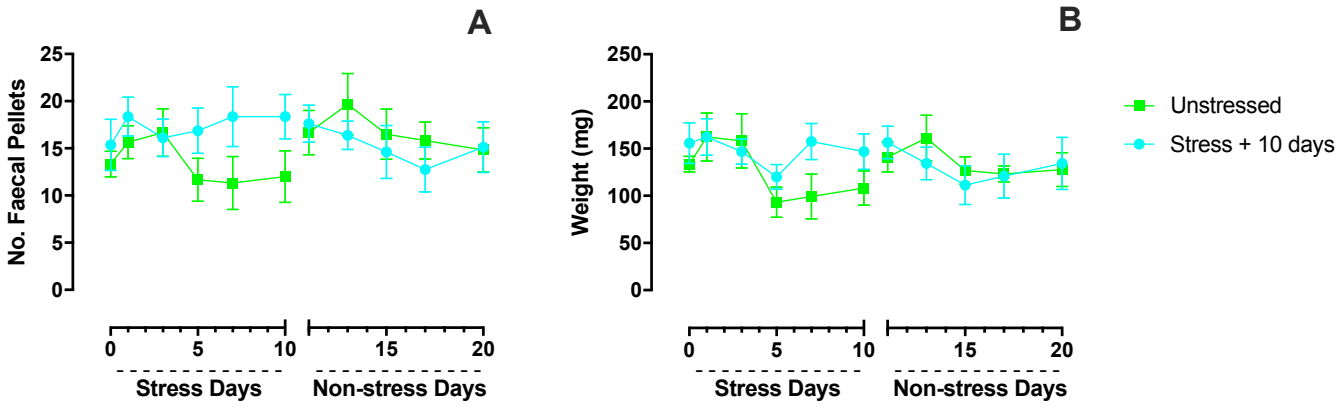

Figure 4.19: Faecal pellet analysis conducted in Stress + 10-days and Unstressed mice. (A) Number of faecal pellets, (B) relative weight of faecal pellets. Datum is presented as mean ± SEM (n = 6). Analysis was performed using two-way repeated measures ANOVA.

Mediator Release

Serosal ATP levels were unchanged across all animal groups **(Figure 4.20.B)**. Overall, the stress-free period of recovery did not affect the release of mediators into the serosal fluid. Similarly, intraluminal levels of ATP and ACh were not significantly altered by 10-days exposure to WAS, nor did 10-days stress-free recovery affect release of these signalling mediators **(Figure 4.20.A and C)**. Release of Ach into the serosal fluid was increased in the Stressed group and increased significantly in the Stress + 10-days group, but only significantly so when compared to the unstressed groups ($p = 0.007$) **(Figure 4.20.D)**.

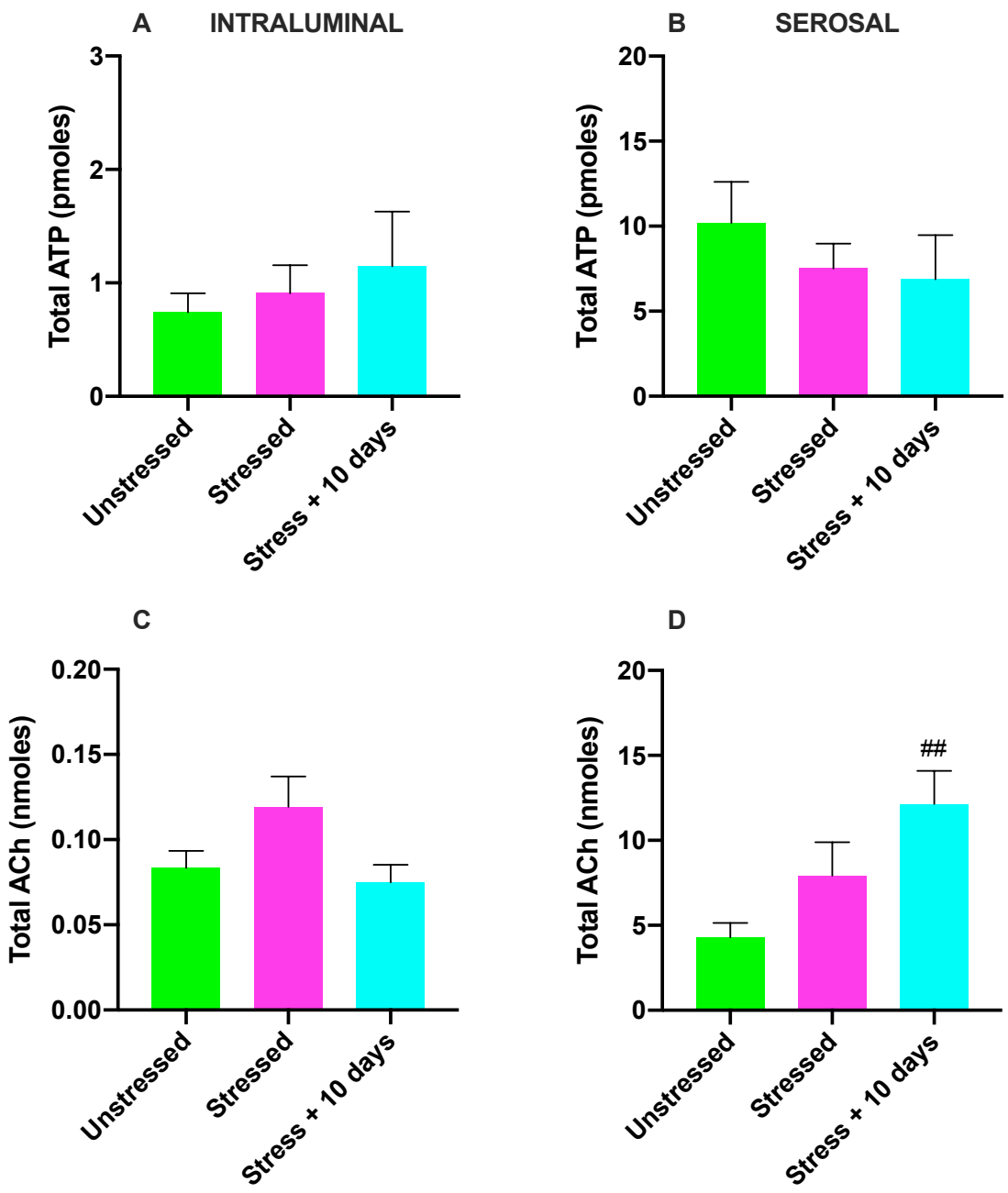

Figure 4.20: Total release of ATP and ACh into the (A & C) intraluminal and (B & D) serosal fluid collected following distensions of isolated bladders in Stressed, Unstressed and Stress + 10-days groups. Datum is represented as mean ± SEM (n = 6). Analysis was performed as a one-way ANOVA ($^{\#\#}p = 0.007$, Stress + 10 day vs. Unstressed).

Bladder Compliance and Stretch-relaxation

Bladder compliance was unchanged in the Stressed group compared to the Unstressed group, just as in part 1 of this chapter. Compliance **(Figure 4.21)** was however, significantly increased in the Stress+10-days group when compared to the unstressed group.

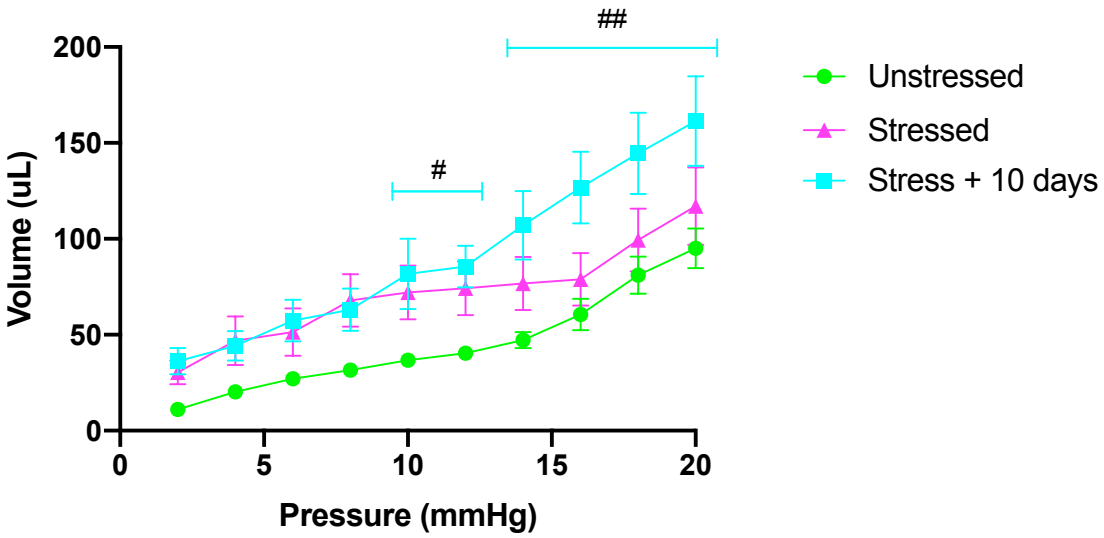

Figure 4.21: Volume-pressure relationship for bladders in Stressed, Unstressed and Stress + 10-days groups. Datum represented as mean ± SEM (n = 6), analysed by two-way ANOVA ($^{\#}p < 0.05$, unstressed vs. stress + 10-days) ($^{\#\#}p < 0.01$, unstressed vs. stress + 10-days).

The fall in intraluminal pressure from 20mmHg over time was also assessed as a measure of compliance and this pressure-time relationship was observed in bladders from the 3 groups to assess relaxation after stretch **(Figure 4.22)**. There was no significant difference in relaxation after stretch between the 3 groups.

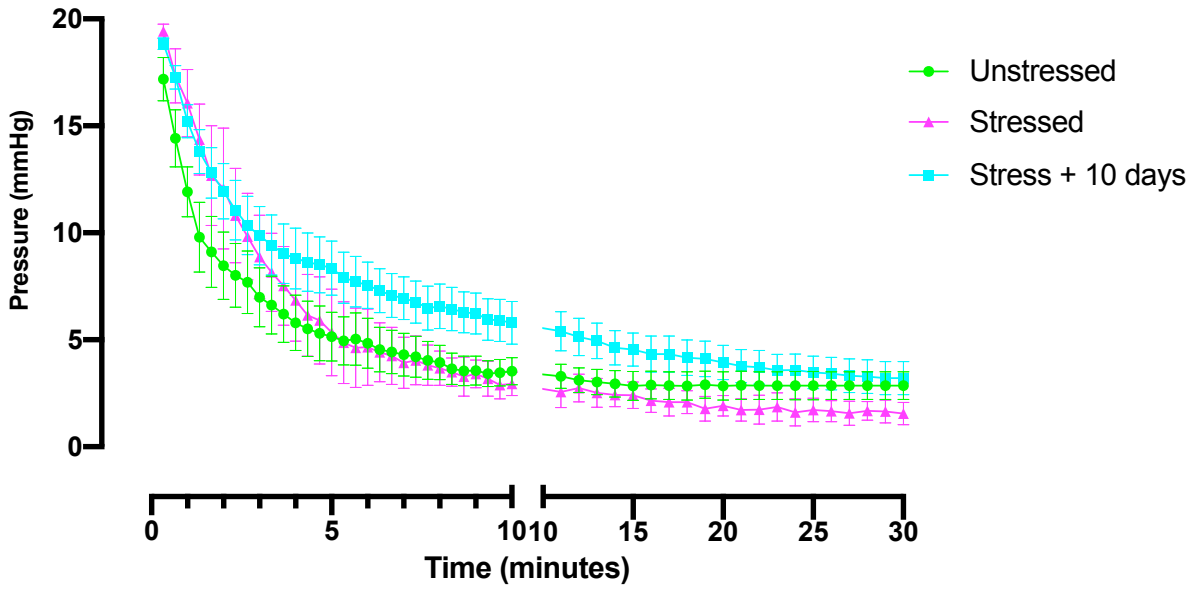

Figure 4.22: Pressure time relationship for bladders from mice in Stressed, Unstressed and Stress + 10-days groups. Datum is represented as mean ± SEM (n = 6), analysed by two-way ANOVA.

Bladder Contractility and Electrical Field Stimulation

There was a significant increase in contractility to KCl in bladders from the stressed group (51.34 ± 3.69 mmHg, n=6), compared to the unstressed group (28.56 ± 5.14 mmHg, n=6), as seen previously in part 1 of this chapter ($p = 0.008$). The response to KCl was significantly reduced back to unstressed levels, in the Stress + 10-days group (27.45 ± 4.01 mmHg, n=6) ($p = 0.006$) **(Figure 4.23)**.

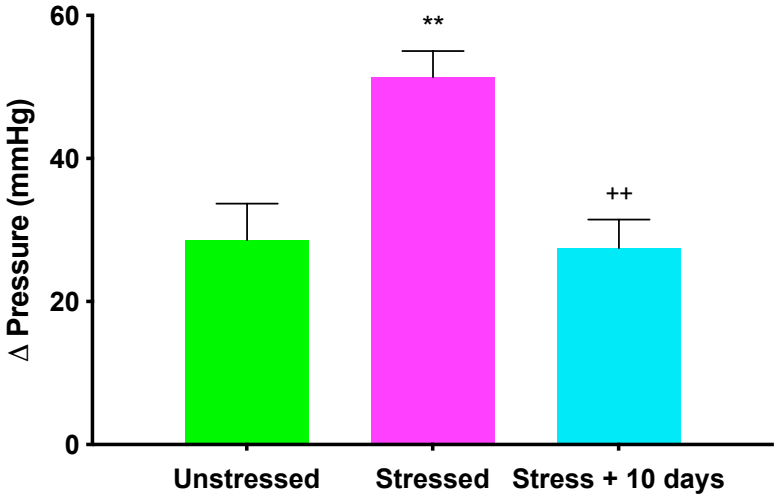

Figure 4.23: Pressure-responses to KCl (60 mM) in bladders from mice in Stressed, Unstressed and Stress + 10-days groups. Datum is represented as mean ± SEM (n = 6) and analysed using an Ordinary one-way ANOVA with Tukey Analysis ($^{++}p < 0.01$, Stress + 10-days vs. Stressed) ($^{**}p < 0.01$, Stressed vs. Unstressed Controls).

Similar to as reported in Part 1, stress did not alter nerve-mediated bladder contractions and the responses to EFS were also not changed in stressed mice following the 10-days stress-free period **(Figure 4.24.A)**. When the EFS responses were normalised to the KCl response, nerve mediated contractions in both stress groups did appear to be depressed compared to the unstressed group, however there was no significant difference between groups **(Figure 4.24.B)**.

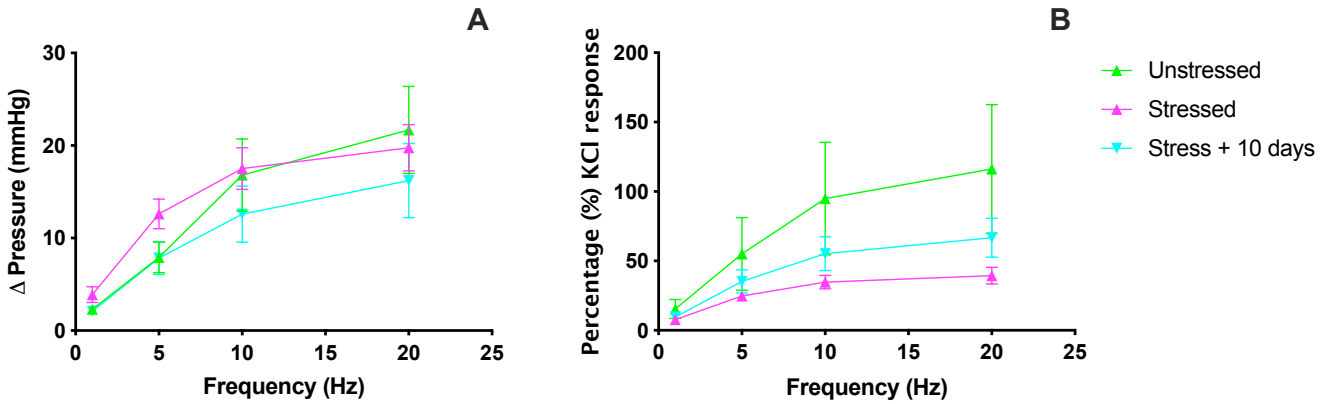

Figure 4.24: Nerve-mediated (1-20 Hz) pressure response of isolated bladders from mice in Stressed, Unstressed and Stress + 10-days groups. Responses were recorded as (A) a change in pressure from baseline and (B) a percentage of the KCl response. Datum is presented as the mean ± SEM (n = 6). Datum analysed using a two-way repeated measures ANOVA with Tukeys comparison.

Electrical field stimulation was repeated at 20 Hz in the absence and presence of several pharmacological agents. The addition of LNNA (100 μM) did not significantly alter bladder contraction to EFS, as seen in the Part 1, indicating that the inhibitory neurotransmitter, NO, was not involved in neurotransmission **(Figure 4.25.A).** Addition of the muscarinic agonist, atropine (1 μM), reduced the response to EFS by 23.96 ± 3.39% (n=6) in Unstressed bladders. A similar reduction was observed in responses in the Stressed and Stress + 10-days group, 26.92 ± 4.88% and 20.91 ± 4.89%, respectively (n=6) **(Figure 4.25.B).** Finally, with addition of αβmATP (1 mM), to desensitise P_2X_1

purinoceptors, responses were further decreased by 51.16 ± 5.04% (n=6) in unstressed controls, 65.69 ± 4.44% in Stressed and 49.75 ± 6.20% in Stress + 10-days groups (n=6) (Figure 4.25.C).

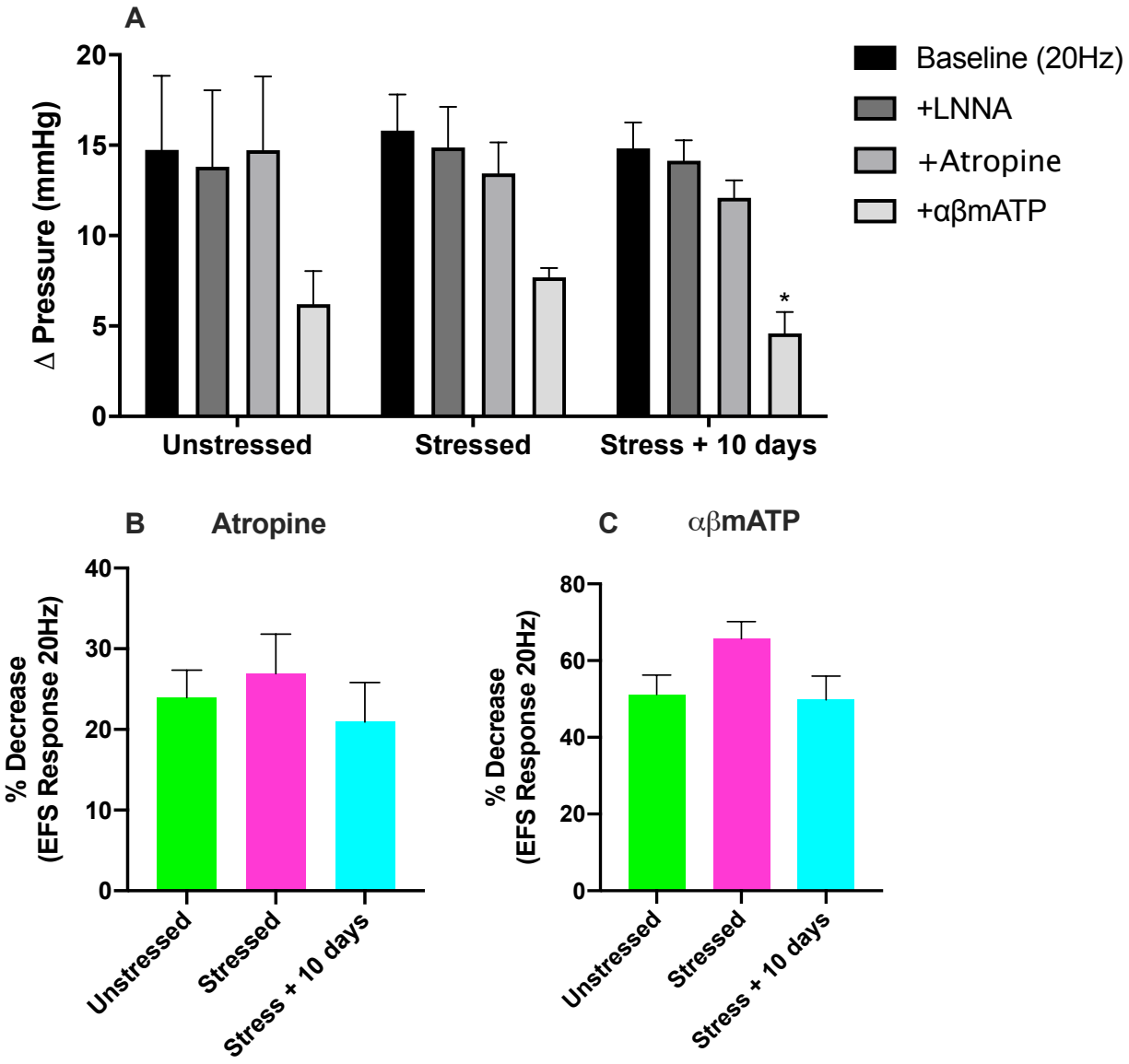

Figure 4.25: A) Intravesical pressure responses to electrical field stimulation at 20 Hz (baseline), and after addition of L-NNA (100 μM), atropine (1 μM) and αβmATP (1 mM) to for bladders from mice in Stressed, Unstressed and Stress + 10-days groups. Percentage decrease in EFS response on addition of (B) atropine (1 μM) and (C) αβmATP (1 mM) to bladder from each group. Datum is expressed as mean ± SEM and analysed using two-way repeated measures ANOVA with Dunnett's multiple comparisons test for (A) and one-way ANOVA with Dunnett's multiple comparisons test for (B and C) (*$p < 0.05$ vs. Baseline (20 Hz)).

Response to Pharmacological Agents

Responses to ATP were similar in bladders from the stressed groups (12.94 ± 1.39 mmHg, n=6), compared to the unstressed group (11.27 ± 1.41 mmHg, n=6), as seen in part 1 of this chapter, but in this case the difference in response was not statistically significant. Responses to ATP were also similar in the stress-free recovered group (9.58 ± 1.441 mmHg, n=6) compared to the unstressed group **(Figure 4.26.A)**. The contractile response was also normalised to KCl response, with no significant difference between the groups **(Figure 4.26.B)**.

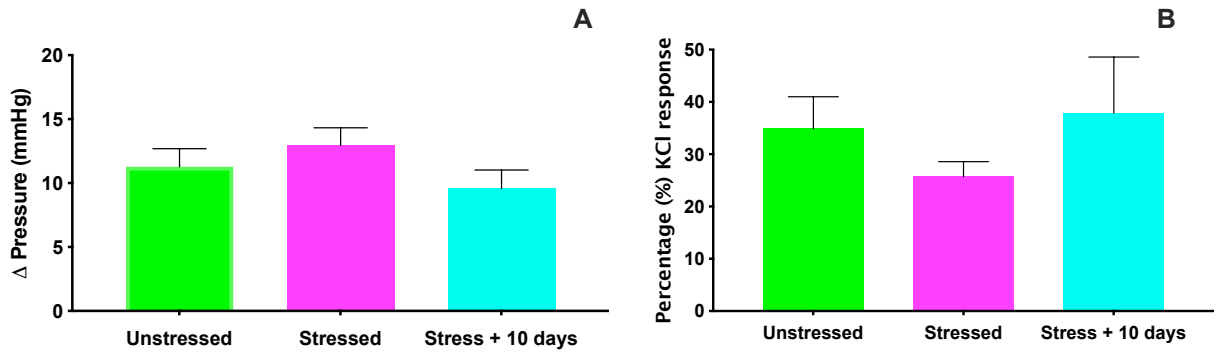

Figure 4.26: Pressure responses to (A) ATP (10 mM) and (B) ATP normalised to KCl response in bladders from mice in Stressed, Unstressed and Stress + 10-days groups. Datum is represented as mean ± SEM (n = 6) and analysed using an ordinary one-way ANOVA with Tukeys comparison.

Contractile bladder responses to αβmATP were also assessed. Compared to unstressed group (15.13 ± 3.41 mmHg, n=6), there was no significant difference in response of recovered (stress + 10-days) (13.5 ± 2.17 mmHg, n=6) and stressed groups (14.7 ± 1.98 mmHg, n=6). The contractile response was also normalised to KCl response, with no significant difference between the groups **(Figure 4.27.A & B)**.

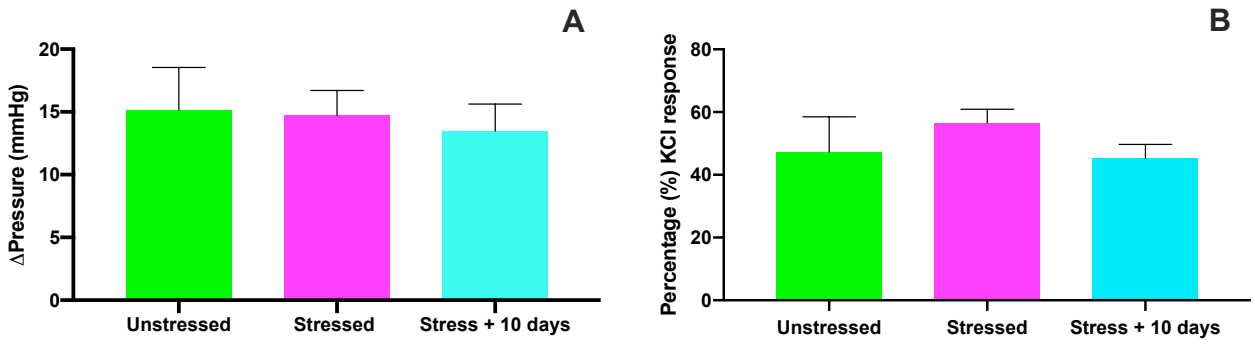

Figure 4.27: (A) Pressure responses to $\alpha\beta$mATP (1 mM) and (B) pressure responses as a percentage of KCl, from bladders in Stressed, Unstressed and Stress + 10-days groups. Datum is represented as mean ± SEM (n = 7) and analysed using an ordinary one-way ANOVA with Tukey comparison.

Carbachol produced a concentration dependent increase in intravesical pressure in bladders from all three groups, with a significantly greater in the maximal response evident in the Stress group compared to Unstressed and Stress+10-days groups **(Table 4.5 and Figure 4.28)**. There was, however, no significant difference in pEC_{50} as highlighted in **Table 4.5**. When the concentration-response curve was normalised as a percentage of the KCl response, no significant difference was observed between the groups. The response to carbachol returned to unstressed control levels in bladders from stressed mice following 10-days stress-free recovery **(Figure 4.28)**. These results reveal that the stress-free recovery period after water avoidance stress returned contractile responses to the muscarinic agonist, carbachol to levels comparable to unstressed control mice.

TABLE 4.5: Whole bladder responses to carbachol in control (unstressed) and water avoidance stress (stressed) and recovered (stress + 10-days) mice (n=6).

	Unstressed	Stressed	Stress + 10-days
pEC_{50}	5.66 ± 0.09	5.53 ± 0.03	5.46 ± 0.09
Maximal response			
ΔPressure (mmHg)	44.41 ± 6.88	56.56 ± 1.91 ($p < 0.05$)	44.73 ± 5.65
Response (% of KCl)	141.11 ± 10.20	112.97 ± 10.96	143.28 ± 23.01

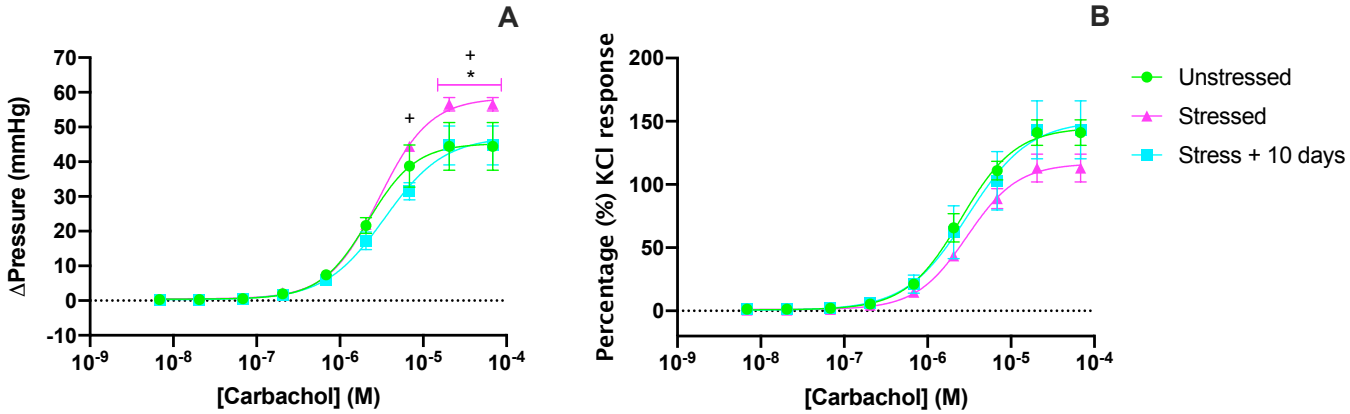

Figure 4.28: Carbachol concentration-response curve for isolated whole bladders from mice in Stressed, Unstressed and Stress + 10-days groups, recorded as (A) change in intravesical pressure from baseline and (B) change in intravesical pressure as a percentage of the KCl response. Datum is represented as mean ± SEM (n = 6), analysed using non-linear regression and two-way ANOVA with Bonferroni's multiple comparisons test (*$p < 0.05$ Unstressed vs. stressed) (⁺$p < 0.05$, stressed + 10-days vs. stressed).

Following carbachol precontraction, bladder relaxation to the beta-adrenoceptor agonist isoprenaline was assessed and responses were not significantly affected by WAS or stress-free recovery **(Figure 4.29.A)**. There was no significant differences in either the IC_{50} values or maximal responses between the groups **(Table 4.6)**. Relaxation responses were also presented as percentage of the carbachol pre-contraction, but again there were no significant differences between the three groups **(Figure 4.29.B)**.

TABLE 4.6: *Whole bladder responses to isoprenaline in control (unstressed), water avoidance stress (stressed) and recovered (stress + 10-days) mice (n=6).*

	Unstressed	**Stressed**	**Stress + 10-days**
pIC_{50}	6.86 ± 0.08	6.81 ± 0.11	6.58 ± 0.15
Maximal response			
ΔPressure (mmHg)	-5.21 ± 0.47	-4.37 ± 0.41	-4.76 ± 0.48
Response (% Decrease)	-104.48 ± 51.14	-51.11 ± 63.92	-77.42 ± 69.88

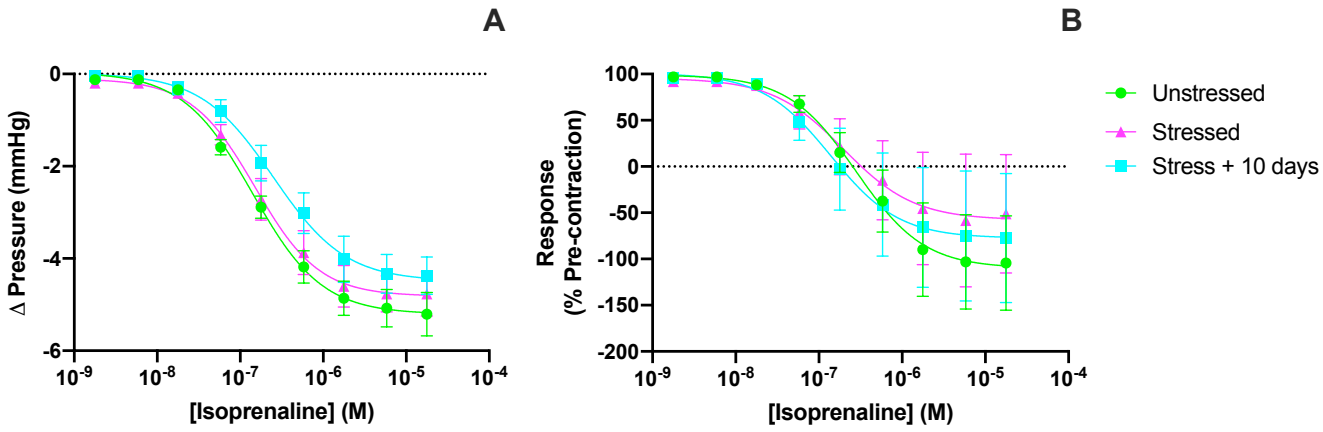

Figure 4.29: Effect of isoprenaline on isolated bladders from mice in Stressed, Unstressed and Stress + 10-days groups with datum given as (A) raw data as change in pressure from pre-contraction, (B) as a percentage (%) of the carbachol precontraction. Datum represented as mean ± SEM (n = 6), analysed using two-way ANOVA and non-linear regression.

Spontaneous Phasic Contractions

Spontaneous activity followed much of the same trend as stated previously in this chapter. During the stretch-relaxation period, the frequency and amplitude of spontaneous activity was not significantly altered by stress when compared to unstressed controls, and 10-days stress-free did not alter these responses **(Figure 4.30.A and B)**.

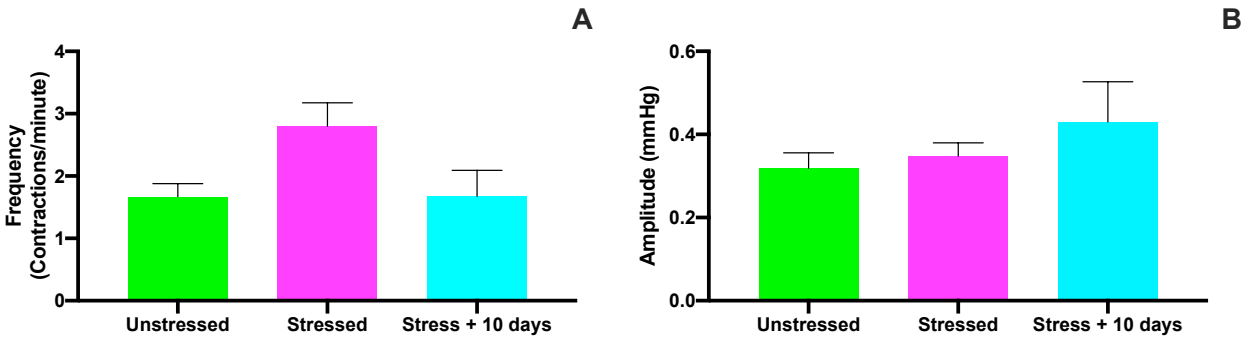

Figure 4.30: Spontaneous activity during stretch-relaxation period in mice from Stressed, Unstressed and Stress + 10-days groups. (A) Frequency of contractions and (B) Amplitude of spontaneous contractions, compared to controls (Unstressed). Datum is represented as mean ± SEM (n = 6), analysed using one-way ANOVA.

Phasic contractions were also measured following addition of 1µM carbachol. The frequency of phasic activity was significantly elevated, as seen previously in part 1 of this chapter ($p = 0.0024$), in bladders from the stressed group (7.00 ± 0.31 mmHg, n=6) compared to the unstressed group (3.66 ± 0.33 mmHg, n=6). Stress-free recovery, Stress + 10-days (4.83 ± 0.79 mmHg, n=6), appeared to reduce the frequency of phasic contractions significantly compared to the stressed group ($p = 0.04$) **(Figure 4.31.A)**. Across all groups however, amplitude remained unchanged **(Figure 4.31.B)**.

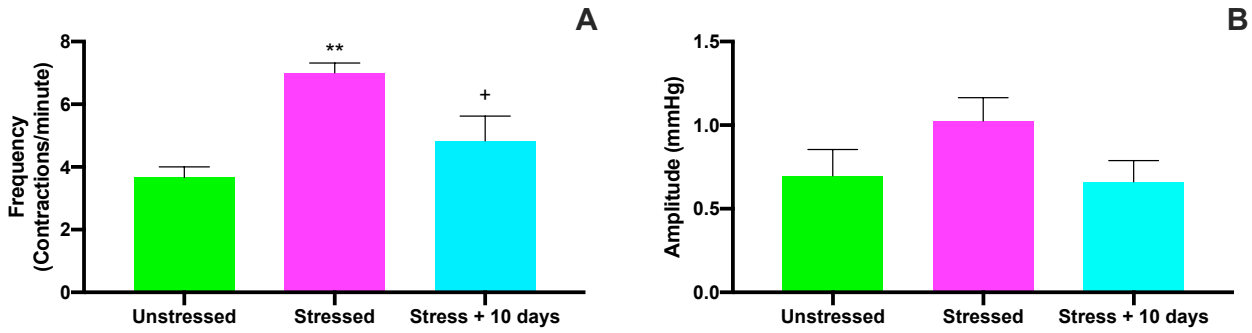

Figure 4.31: Phasic response to tonic contraction of 1 μM carbachol in Stressed, Unstressed and Stress + 10-days groups. (A) Frequency of phasic contractions and (B) Amplitude of phasic contractions. Datum is represented as mean ± SEM (n = 6), analysed using a one-way ANOVA (**$p < 0.01$, Unstressed vs. stressed) ($^{+}p < 0.05$, stressed + 10-days vs. stressed).

DISCUSSION

There is abundant clinical evidence that psychological stress is associated with bladder dysfunction and pathologies. Currently, there is a lack of understanding of the local bladder changes that occur due to psychological environmental stress. This study employed an animal model of water avoidance stress to determine the impact of environmental stress on voiding behaviour and bladder function and establish the role that recovery plays in this dysfunction.

Effects of Stress on Voiding Behaviour

In this study we observed a time-dependent increase in voiding frequency in water avoidance stressed mice compared to the unstressed mice, with a significant change as early as day 3 of the stress protocol. Yoon, Lee, Chun, Yoon, and Yoo (2010) also observed a similar increase in voiding frequency, which increased over time , however, they reported that stress also increased the volume of urine output compared to the control animals. Our study found no change in urine output or water consumption, indicating that the increase in voiding frequency caused by stress is due to changes in local and/or central micturition control mechanisms and not excess water consumption/urine production over the time points tested. The number of small voids increased significantly in the stressed group, while the average void size decreased; suggesting that voiding interval decreased in the stressed group, similar to previously observed changes in frequency, latency to void, voiding interval and volume in stressed versus control mice (Smith et al., 2011). These findings suggest that stressed mice are urinating a smaller amount, more frequently than the unstressed mice which indicates that the bladder may not be emptying completely on each void or that micturition is being initiated at a lower bladder volume.

Evidence from the literature suggests that there are sex differences in voiding dysfunction following water avoidance stress. In our model of water avoidance stress, an increase in voiding frequency was observed in female mice, similar to the changes reported by Smith et al. (2011) in female rats. However, McGonagle et al. (2012), reported a decrease in voiding frequency in male mice following water avoidance stress. Studies looking at other models of psychological stress have found the same trend with gender (Mann, 2015; West, Sellers, Chess-Williams, & McDermott, 2020).

Effects of Stress on Corticosterone Release

Exposure to water avoidance stress produced a significant hormonal stress response in the stressed mice, with elevated plasma corticosterone levels evident following 10-days stress exposure. This confirmed that the mice used in this study were stressed when the voiding pattern analysis and whole bladder preparations were performed. This is consistent with other animal studies which have observed increased corticosterone levels in water avoidance stressed mice (Hassan et al., 2014; Wang et al., 2017). While it is well documented that chronic and acute stress stimulate the release of stress hormones, such as cortisol in the human and corticosterone in the rodent, the exact effect of the hormone on the bladder has thus far been undocumented.

In a rat model of water avoidance stress, it has been observed that chronic stress is associated with degenerative changes to the urothelial layer of the bladder (Cetinel, Ercan, Cikler, Contuk, & Sener, 2005). Research has linked this change in the bladder to an increase in cyclooxygenase 2 (COX2) expression and subsequent release of pro-inflammatory cytokines (Yamamoto et al., 2012). There are two isoforms of cyclooxygenase, COX1 and COX2 with arachidonic acid being the substrate for both forms (Ricciotti & FitzGerald, 2011). COX2 in particular, is responsible for the production of

prostaglandin E_2 (PGE_2) which causes inflammation and pain **(Figure 4.32)** (Ong, Lirk, Tan, & Seymour, 2007). Interestingly, the same study also observed an increase in voiding frequency in stressed mice, just like the results from this chapter. Previously, our group has reported on an association between an increase in urinary frequency with increased COX2 production, due to inflammation (West et al., 2018). A study looking at the role of cyclooxygenase in the HPA axis, found that there was considerable participation of COX1 and COX2 in the stimulating of β_2-adrenergic receptor-mediated function of the HPA axis (Bugajski, Glod, Gadek-Michalska, & Bugajski, 2001). This increase has also been assessed in aged rats and it was found that increased pro-inflammatory cytokines target hypothalamic receptors of the HPA axis to stimulate corticotropin secretion (Sapolsky, Rivier, Yamamoto, Plotsky, & Vale, 1987). Therefore, a suspected increase in COX2 and subsequent release of inflammatory cytokines onto the HPA axis, may play a role in stress response and altered voiding in water avoidance stressed mice.

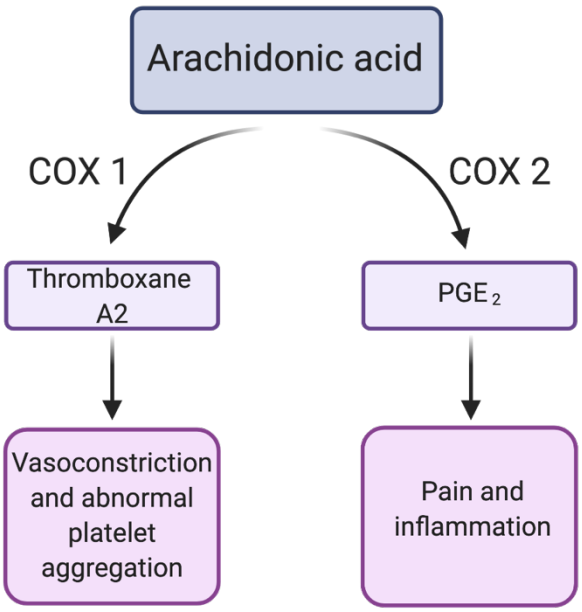

Figure 4.32: Production of Cyclooxygenase (COX). Flow chart representing the actions of COX 1 and COX 2 from arachidonic acid which leads to further production of PGE_2 (pro-inflammatory cytokine), leading to pain and inflammation. (Created with BioRender.com by the author)

Effects of Stress on Bladder Physiology

To investigate the local bladder mechanisms that may contribute to the altered voiding phenotype observed in the stressed mice, bladder compliance, urothelial signalling mediators and contractile responses were assessed using a whole-bladder preparation. Nerve mediated bladder responses to EFS, at all frequencies, were not affected by stress. Similarly, there was no change observed in the contribution of ACh or ATP to nerve-mediated responses to EFS or in the beta-adrenoceptor mediated bladder relaxation. However, contractile responses to muscarinic (carbachol) and receptor-independent (KCl) detrusor stimulation, as well as responses to αβmATP and ATP were increased significantly in bladders from the stressed group. This suggests that an overall increase in detrusor contractility rather than changes in receptor expression and may instead, be related to Rho-kinase and calcium sensitisation (Frazier, Peters, Braverman, Ruggieri, & Michel, 2008).

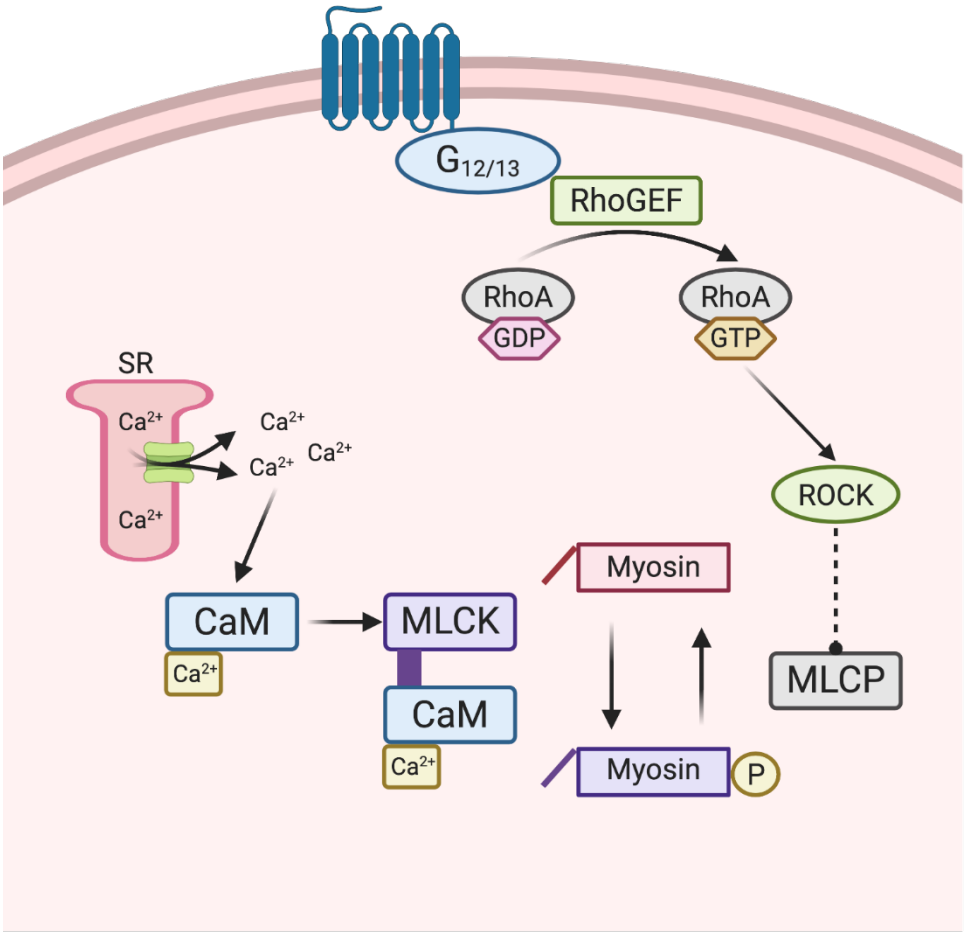

Figure 4.33: Intracellular mechanisms involved in smooth muscle contraction. Calmodulin (CaM), Calcium (Ca^{2+}), Guanosine diphosphate (GDP), Guanosine triphosphate (GTP), G protein-coupled receptor alpha subunits 12/13 ($G_{12/13}$), Myosin light chain kinase (MLCK), Myosin light chain phosphatase (MLCP), Roh-associated protein kinase (ROCK), Sarcoplasmic reticulum (SR). (Created with Biorender.com by the author).

Changes in intracellular calcium have been implicated in various smooth muscle related disorders. Smooth muscle contraction in the detrusor is driven by free intracellular calcium concentration and phosphorylation of a number of enzymes, with smooth muscle contraction being dependent on the phosphorylation state of myosin light chain kinase (MLCK). Studies have also demonstrated that smooth muscle contraction can occur independently of calcium concentration by inhibition of myosin phosphatase (MLCP) by Rho-associated protein kinase (ROCK) (Somlyo & Somlyo, 2000; Yoshii et al., 1999).

There are three isoforms of Rho-kinase that make up the GTPase family, RhoA, RhoB and RhoC. RhoA however, is the best understood isoform of Rho-kinase due to its involvement in smooth muscle contraction (de Godoy & Rattan, 2011) **(Figure 4.33)**. RhoA is part of a molecular switch which changes from active guanosine triphosphate (GTP)-bound to inactive guanosine diphosphate (GDP)-bound (Yoshii et al., 1999). This switch is activated by Rho-specific guanine nucleotide exchange factor (RhoGEF). Once converted to RhoA GTP-bound, interactions occur between multiple effectors including ROCK (Wettschureck & Offermanns, 2002). ROCK, once activated by RhoA, phosphorylates the myosin binding subunit, leading to inactivation of MLCP. Inhibition of MLCP leads to increased activity of myosin phosphorylation which acts to induce actin-myosin cross-bridging causing smooth muscle contraction (Somlyo & Somlyo, 2000). There has also been evidence of ROCK directly phosphorylating MLC_{20}, which activates myosin to form cross-bridges with actin filaments, causing contraction (Somlyo & Somlyo, 2000). Therefore, ROCK causes calcium sensitisation and enhances smooth muscle contraction independently of calcium concentration.

The ROCK pathway has been shown to be upregulated under pathophysiological conditions in bladder smooth muscle (Zhang & DiSanto, 2011). One study examined the changes in Rho-kinase after environmental stress of female rats (Yoon et al., 2010), performing immunohistochemical staining for ROKα, the activating enzyme of ROCK. The study found that there was an increase in ROKα expression in the bladders of stressed rats compared to the controls and this was accompanied with an increase in voiding frequency with stress (Yoon et al., 2010). Chronic variable stress also increased RhoA/ROCK expression in the rat bladder, with a decrease in micturition duration, interval and volume also reported (Han, Jeong, & Lee, 2015). The changes in the ROCK pathway previously described, may explain the overall enhanced bladder contractility of

the stressed mice in our study, and may contribute to alterations in voiding behaviour with water avoidance stress.

Interestingly, nerve mediated bladder responses to EFS stimulation were not affected by stress, even though general contractility was increased. As stated previously, normal physiological contraction occurs by the co-release of neurotransmitters ACh and ATP, acting on M_3 muscarinic receptors and P_2X_1 receptors, respectively. As seen above, the relative contribution of these neurotransmitters was not altered by stress in the present study. As an increase in general contractility was observed in the stressed group, the absence of changes in nerve mediated contraction suggests that the process of neurotransmission and/or the efferent nerves were affected by stress. Many studies have reported increased nerve density in overactive bladder (Arrabal-Polo et al., 2012), however, our findings would not support this. Instead, it may be suggested that there is an increased breakdown of neurotransmitters after repeated psychological stress. While this theory has not been well documented previously, one study investigated the effect of acute and chronic restraint stress on ATP hydrolysis in hippocampal synaptosomes of male rats. The study observed that ATP diphosphohydrolase activity increases with acute stress, contributing to the elimination of ATP and an increase in the availability of extracellular adenosine. Chronic stress, however, resulted in increased ecto-ATPase (Fontella et al., 2004), which works to catalyse the hydrolysis of extracellular ATP to ADP and inorganic phosphate (Dombrowski, Ke, Brewer, & Kapp, 1998). A_1 adenosine receptors have been implicated in modulating neuromuscular transmission in the detrusor muscle. One study has observed selective A_1 adenosine receptor agonists on the contractile responses of detrusor muscle preparations to EFS and found that A_1 agonists inhibited murine bladder contraction in response to EFS (Searl et al., 2016). Greater

neurotransmitter breakdown may explain why the nerve-mediated contractions were unchanged in the stress group, despite an increase in bladder contractility.

A sub-maximal carbachol concentration produced a large tonic bladder contraction, followed by spontaneous phasic responses. Both amplitude and frequency of the phasic responses were significantly enhanced in bladders from the stressed group. A number of theories exist as to why spontaneous activity occurs. For instance, muscarinic receptor subtypes, M_2 and M_3, have been linked to the phasic component of carbachol contractions in guinea pig bladder, however, some studies have observed that bladder volume influences the response in the murine bladder (Lagou, Gillespie, Andersson, Kirkwood, & Drake, 2006; Srinivasan, Kim, Burbach, Ford, & Bhattacharya, 2006). It has been previously reported that spontaneous contractions may occur in a murine model of bladder overactivity (McCarthy et al., 2009; West et al., 2018). PGE_2 has been reported to be increased in some overactive bladder conditions and has the ability to increase phasic contractions of the detrusor muscle. PGE_2 has been shown in previous studies to increase calcium transients thereby increasing the frequency of spontaneous depolarisation (Kobayter et al., 2012). Other studies of detrusor smooth muscle have observed that spontaneous contractions are insensitive to tetrodotoxin, thereby confirming the non-neurogenic origin of the contractions, which fits the profile of our study (Drake et al., 2018). Several studies have associated ROCK with the regulation of detrusor smooth muscle tone and therefore if there is an increase in ROCK (Wibberley, Chen, Hu, Hieble, & Westfall, 2003), as suspected due to the general increase in contractility, there would naturally be an increase in bladder tone (Shiomi et al., 2013). Spontaneous phasic activity has been linked with the regulation of bladder tone (Drake et al., 2017). As tone may be increased in the stressed bladders, due to increased ROCK expression, this may be the reason that increased spontaneous activity was observed in

stressed bladders. Given that bladder compliance was not altered by stress, but there was an increase in urinary frequency, it suggests that sensory changes, particularly in spontaneous activity, may be involved in altered voiding patterns observed here.

The results presented here indicate that psychological stress affects local bladder function. The general increase in contractility, potentially due to upregulation of the Rho-kinase calcium sensitisation pathway, may contribute to the increase in urinary frequency and decreased void size observed in the water avoidance stressed mice. In conclusion, functional bladder changes were observed in stressed mice and may be linked to overall increased contractility of the bladder, independent of neuronal stimulation.

Effects of Stress-free Recovery on Stress Induced Changes to Voiding Behaviours and Plasma Corticosterone

To our, knowledge there are few studies which investigate the long-term effects of stress of voiding behaviour and persistence of these changes once stress exposure ends. In this study, voiding analysis took place over a period of 10-days stress-free following exposure to water avoidance stress. The aim was to determine if there is recovery from the initial voiding dysfunction seen in the water avoidance stress model or if further changes occur. Voiding increased over the 10-days stress exposure, as seen in part 1 using the water avoidance stress model in this chapter. While voiding frequency did decrease, over the following 10-days stress-free period, voiding frequency still remained significantly elevated compared to unstressed controls, with an apparent spike at day 20. The same trend was also observed in the number of small voids ($<0.2cm^2$), where the amount increased over the first 10-days and decreased over the stress-free period, with a significant increase at day 20. This demonstrates the long-term effect of psychological stress on bladder function. We are aware of only one experimental study that

investigated the recovery of voiding function following chronic stress exposure, which reported that changes in micturition persisted for approximately 1 month in female rats following WAS, with a mean duration of 24 days, although surprisingly this particular data was not presented in the paper (Smith et al., 2011). Clinical studies have also observed a positive correlation between perceived stress levels and the incontinence symptoms observed in overactive bladder and interstitial cystitis/bladder pain syndrome (Lai, 2015). These findings demonstrate that psychological stress may have a long-term impact on voiding dysfunction, which may fluctuate depending on perceived stressors.

Corticosterone levels have been documented to increase in a number of rodent stress models (Hassan et al., 2014; Wang et al., 2017). Here we demonstrate that corticosterone levels decreased in the stressed mice after 10-days of stress-free recovery, returning to control levels. This indicates that the hormonal response to psychological stress recovers, however this does not necessarily correlate with recovery of bladder function. Several experimental studies look at corticosterone levels in both a chronic and acute stress scenario. One study using a model of social defeat stress observed that chronic stress may cause short-term adaptation in the HPA axis resulting in a maladaptive stress response. The study reported a biphasic corticosterone response where after 2 weeks, a 'switch' occurs to desensitise CRF expression and instead elevate AVP (Keeney et al., 2006). Another study observing repeat restraint stress observed that desensitisation of CRH occurs after 2 weeks of stress and therefore the hypothalamus is flexible to homotypic stress (Ma, Lightman, & Aguilera, 1999). Clinically, studies on humans have also observed a switch from CRF to AVP drive on the HPA axis, which may play a role in the pathophysiology of clinical depression (Scott & Dinan, 2002). This may explain why, after the water avoidance stress period and 10-days of recovery, corticosterone hormone

levels are significantly decreased in the 'recovered' group, compared to the stressed group which had no recovery time.

Effects of Stress-Free Recovery on Mediator Release

Interestingly, serosal ACh was increased in the stressed group and significantly increased in the 10-day stress-free group. This was not seen in the original WAS study, where there was no change in serosal ACh release, and this can only be attributed to the fact that both studies were undertaken a year apart from one another. Due to there not being any change in the contribution of ACh to nerve mediated activity after the addition of atropine to the bath, discussed later, it can be assumed that this increase in ACh was from a non-neuronal source. Studies of non-neuronal ACh release have found that urothelial cells express high-affinity CHT1 as well as ChAT and CarAT (Hanna-Mitchell et al., 2007). While vesicular release of ACh occurs in nerve terminals, non-vesicular release has been shown to occur from the urothelium, due to the absence of the vesicular acetylcholine transporter (VAChT). The same study also identified organic cation transporters (OCT), OCT1 and OCT3, which are important for the release of ACh (Lips et al., 2007).

The role of ACh is still being identified, however, there is some evidence that the neurotransmitter influences sensory nerve activity to stimulate the release of UDIF (Hawthorn et al., 2000). There is limited literature on the effect of recovery after water avoidance stress and therefore it could be theorised that UDIF acts to inhibit detrusor muscle contraction (Templeman, 2002), Therefore, enhanced ACh release, observed in the stress-free recovery group, may act to decrease contractility that occurred within the group. During the storage phase, stretch of the urothelium initiates release of mediators (Birder, Kanai, Cruz, Moore, & Fry, 2010). As there was increased compliance in the

stress-free recovery group, it is also possible that this has caused an increased release of ACh from the urothelium.

Effects of Stress-Free Recovery on Stress Induced Changes to Bladder Physiology

Part 1 of this chapter reported an increase in general contractility after 10-days of water avoidance stress. This increase in contractility caused by stress appears to recover to unstressed control levels following 10-days stress-free. Nerve mediated bladder responses to EFS, at all frequencies, were not affected by recovery. Similarly, there was no change observed in the contribution of ACh or ATP to EFS-induced responses or in the beta-adrenoceptor mediated bladder relaxation. Contractile responses to muscarinic receptors (carbachol) and receptor-independent (KCl) detrusor stimulation as well as response to αβmATP and ATP were also reduced in the recovered group compared to the stressed group. Sub-maximal tonic contraction to carbachol was also reduced in the recovered group and the frequency of the subsequent phasic activity was reduced, compared to the stressed group. These changes indicate that there is some recovery of the bladders after chronic water avoidance stress. The most significant change observed, however, was in the compliance of bladders from stressed mice following 10-days stress-free period, which increased significantly compared to both the bladders from unstressed and stressed mice. This suggests that a stress-free recovery period initiated a form of compensation, increasing bladder compliance, thereby reducing the impact of psychological stress on voiding.

The impact of stress on bladder compliance has not been well documented, however, one study observed nociceptive responses in chronic psychological stress. The study found that water avoidance stress causes bladder hypersensitivity meaning that voiding occurs at lower bladder pressures. This may explain why, with increased compliance in the

recovered group, voiding frequency decreased (Gao, Zhang, Chang, & Rodriguez, 2018). Changes in compliance may also manifest with long term changes in bladder morphology. Bladder fibrosis is known to cause changes to both the detrusor muscle and extracellular matrix (Lluel et al., 2000). The extracellular matrix of the bladder is made up of Type I and Type III collagen. Type I collagen helps to maintain tension and smooth muscle binding, leading to decreased contractility and compliance (Inaba et al., 2007). Type III collagen increases bladder elasticity and contractility of smooth muscle (Stevenson, Kucich, Whitbeck, Levin, & Howard, 2006). One study has found that after chronic stress of female mice, both types I and III collagen were increased in bladders, which demonstrates that stress can induce pathological changes in bladder stability (Yoon et al., 2010). It may be hypothesised that changes in collagen, due to stress, may continue to increase or may not recuperate to unstressed control levels, in the 10-days after stress, thereby increasing compliance in the recovered bladders. This could be investigated in future studies, by performing histology and looking at markers of Type I and Type III collagen.

The long-term changes in bladder collagen may also explain why symptoms of overactive bladder reoccur with fluctuations of psychological stress. Increased collagen has been linked to detrusor overactivity in a number of clinical studies and has been linked to detrusor overactivity and urinary retention in men (Bellucci et al., 2017), and stress urinary incontinence in women (Kushner, Mathrubutham, Burney, Greenwald, & Badlani, 2004).

The persistent elevation in voiding frequency following a stress-free period, despite increased bladder compliance and recovery of detrusor contractile responses suggests that there may also be sensitization of bladder afferent nerves and/or alterations to micturition control centre contributing to the stress induced voiding dysfunction.

CONCLUSION

The results presented here indicate that psychological stress affects bladder function, evident in the increased voiding frequency and increased contractile responses. There was a non-specific increase in detrusor contraction which appears to be offset by the efferent innervation/neurotransmission. Furthermore, urothelial release was not significantly changed following WAS at the level of distension tested. A period of recovery appeared to reduce the increased contractility induced by stress and was associated with enhanced compliance. Interestingly, while voiding frequency was reduced following stress-free recovery, it remained elevated compared to unstressed controls which indicates another underlying mechanism may be responsible, possibly involving sensory nerves.

CHAPTER 5: EFFECTS OF SERTRALINE ON BLADDER DYSFUNCTION

The results presented in this chapter have been published: West, Eliza G., Sellers, Donna J., Chess-Williams, Russ., McDermott, Catherine. (2021). "The anxiolytic sertraline reduces the impact of psychological stress on bladder function in mice", *Life Sciences*, 2021, 119598, https://doi.org/10.1016/j.lfs.2021.119598.

INTRODUCTION

Depression and anxiety are major contributors to the global burden of disease. The management of anxiety and depression is through primary care and often the administration of SSRI antidepressants (Lewis et al., 2019). One of the most prescribed SSRI's on the market is sertraline, clinically known as Zoloft (Hillhouse & Porter, 2015) and is the SSRI used in the current study.

Serotonin and Selective Serotonin Reuptake Inhibitors (SSRI)

Serotonin is a neurotransmitter that impacts several processes, including motor activity, hormone secretion, cognition, and autonomic function. The activation of serotonin neurons is controlled by several efferent pathways including glutamatergic input in the prefrontal cortex, tonic noradrenergic input from pontine nuclei and inhibitory GABAergic input from interneurons. Within the terminal axon of the serotonergic neuron, a complex cascade begins with tryptophan converting to serotonin (5-HT), when stimulated by the aforementioned processes (Adell, Celada, Abellan, & Artigas, 2002).

5-HT is accumulated and stored within presynaptic vesicles. Release of serotonin occurs from the presynaptic vesicles into the synaptic cleft when an action potential stimulates the neuron. The released 5-HT can interact with both post and pre-synaptic receptors, which results in attenuation or stimulation of further release (Umehara et al., 2016). Postsynaptic receptors (5-HTR1 and 5-HTR2) work together to activate second

messenger cascades (Struder & Weicker, 2001), with the main signalling pathway, of 5-HTR1, being coupling via G proteins to decrease cyclic AMP and inhibit adenylate cyclase. The signalling pathway of 5-HTR2 activates phospholipase C, again via G protein coupling, thereby catalysing the formation of IP3 and DAG (Raymond et al., 2001). These pathways then activate neurotransmitter release from serotonergic, noradrenergic, and dopaminergic neurons. Serotonin that does not attach to receptors is transported back into the presynaptic terminals via the membrane protein, solute carrier family 6 member 4 (SCL6A4). SCL6A4 is part of the Na^+/Cl^- dependent transporter family (Quick, 2003). See **Figure 5.1** for a diagram of this pathway.

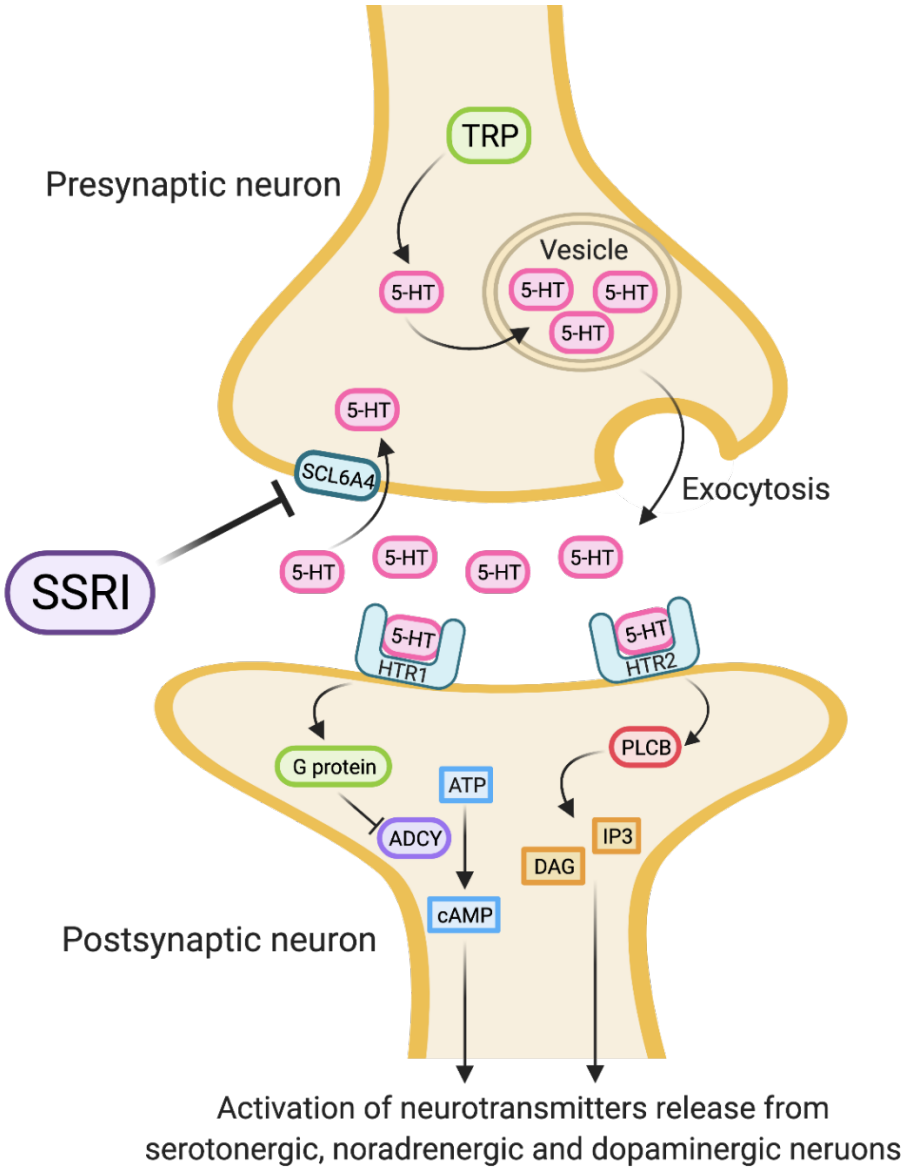

Figure 5.1: Serotonergic neuron displaying cascade from serotonin (5-HT) production in presynaptic neuron to stimulation of postsynaptic neuron and subsequent activation of neurotransmitter releases from serotonergic, noradrenergic, and dopaminergic neurons. Tryptophan (TRP), Postsynaptic neuron receptors (5-HTR1 and 5-HTR2), Adenylyl cyclase (ADCY), Adenosine Triphosphate (ATP), Cyclic AMP (cAMP), Phospholipase C (PLCB), Inositol triphosphate (IP3) and Diacylglycerol (DAG). (Created with Biorender.com by the author).

SSRIs were developed over several years to target specific serotonin uptake sites with high affinity. SSRIs also have a low affinity to noradrenaline uptake sites and very low affinity to other neurotransmitters uptake sites (Hiemke & Hartter, 2000). SSRIs have been used to treat both major clinical and minor depression and have also been used to

treat other psychiatric disorders associated with serotonin system dysfunction (Denboer, Westenberg, Deleeuw, & Vanvliet, 1995). SSRIs block reuptake of serotonin back into the presynaptic neuron, thereby increasing the amount of serotonin available in the synapse. The serotonin therefore has more opportunity to attach to 5-HTR1 and 5-HTR2 receptors, as seen in **Figure 5.1** (Hiemke & Hartter, 2000). Ascending serotonin neuron projections terminate in several regions including the cortical, limbic, midbrain and hindbrain regions. These regions that are modulated by serotonin production are responsible for mood, appetite and memory, amongst others, which explains why drugs targeting specific serotonin receptors have effects on multiple behavioural processes (Giorgetti & Tecott, 2004).

5-HT Receptors Involved in Bladder Function

As discussed in Chapter 1, normal bladder function relies on a coordinated pattern of relaxation and contraction by excitatory and inhibitory nerve activity, controlled predominately by the pons micturition centre and forebrain (Khaled & Elhilali, 2003). Three subtypes of 5-HT receptors, 5-HT_1, 5-HT_2 and 5-HT_3, are found to be present in various areas of the lumbosacral spinal cord known to innervate the bladder (Monroe & Smith, 1983). These areas include the dorsal horn, the site of bladder afferent nerve terminals (Monroe & Smith, 1983), the sacral parasympathetic nucleus, the site of efferent neurons to the bladder (Thor, Nickolaus, & Helke, 1993) and the Onuf nucleus, the site of efferent neurons to the external urethral sphincter (Helton, Thor, & Baez, 1994). While the role of serotonin as a key neurotransmitter in the central nervous system has been well established, 5-HT has also been documented to play an important role in LUT function (Nishizawa, 2015).

In addition to 5-HT actions within the lumbosacral spinal cord, 5-HT also acts on autonomic excitatory efferent nerve terminals. 5-HT_{2A} and 5-HT_{2C} receptors in guinea pig and rat bladder, respectively, have been shown to enhance nerve mediated contraction. These subtypes were also found to facilitate purinergic transmission in autonomic efferent nerves (Inoue, Kitazawa, Cao, & Taneike, 2003). Another, more controversial study has suggested that 5-HT_4 enhances ACh release from autonomic efferent nerve terminals in human detrusor (Candura et al., 1996). In contrast to this, the 5-HT_{1A} subtype has been reported to have an inhibitory action on nerve evoked ACh release in human detrusor (D'Agostino, Condino, Gallinari, Franceschetti, & Tonini, 2006) **(Figure 5.2)**.

Within the bladder itself, a number of 5-HT receptors have been identified, however the effects of 5-HT on voiding are poorly understood due to multiple sites of action and several receptor subtypes. Within the detrusor muscle, 5-HT receptor subtypes 5-HT_{1A}, 5-HT_2, 5-HT_3 and 5-HT_7, have been identified at post junctional detrusor sites (Matsumoto-Miyai, Yoshizumi, & Kawatani, 2015). Across different species, 5-HT_2 subtypes have been shown to be involved with postjunctional detrusor smooth muscle contraction, particularly in dogs (Cohen & Tromba, 1989), human (Klarskov & Horby-Petersen, 1986) and rats (Sakai, Kasahara, Tomita, Ikegaki, & Kuriyama, 2013). Stimulation of the 5-HT_{1A} receptor has also been shown to produce a reduction in cyclic AMP (cAMP) levels, thereby increasing detrusor muscle contraction (Mittra, Malhotra, Naruganahalli, & Chugh, 2007). However, another study of rat bladder strips found that stimulation of 5-HT_3 receptors inhibited detrusor contraction by direct muscle stimulation, but enhanced nerve stimulated contraction (Chetty, Coupar, Chess-Williams, & Kerr, 2007). In terms of EFS-evoked contractions, stimulation of 5-HT_7 receptors facilitates detrusor contraction and interestingly appear to facilitate the release of ACh

from parasympathetic nerve terminals in human bladder (D'Agostino et al., 2006) **(Figure 5.2)**.

As discussed in previous sections, distension of the bladder wall evokes release of ATP from the urothelium via a downstream release of Ca^{2+} from the endoplasmic reticulum (Matsumoto-Miyai, Kagase, Murakawa, Momota, & Kawatani, 2009). There have been studies which suggest that 5-HT reduces urothelial ATP release during distension, mediated by the 5-HT_{1D} receptor subtype (Matsumoto-Miyai, Yamada, Yoshizumi, & Kawatani, 2012) **(Figure 5.2)**.

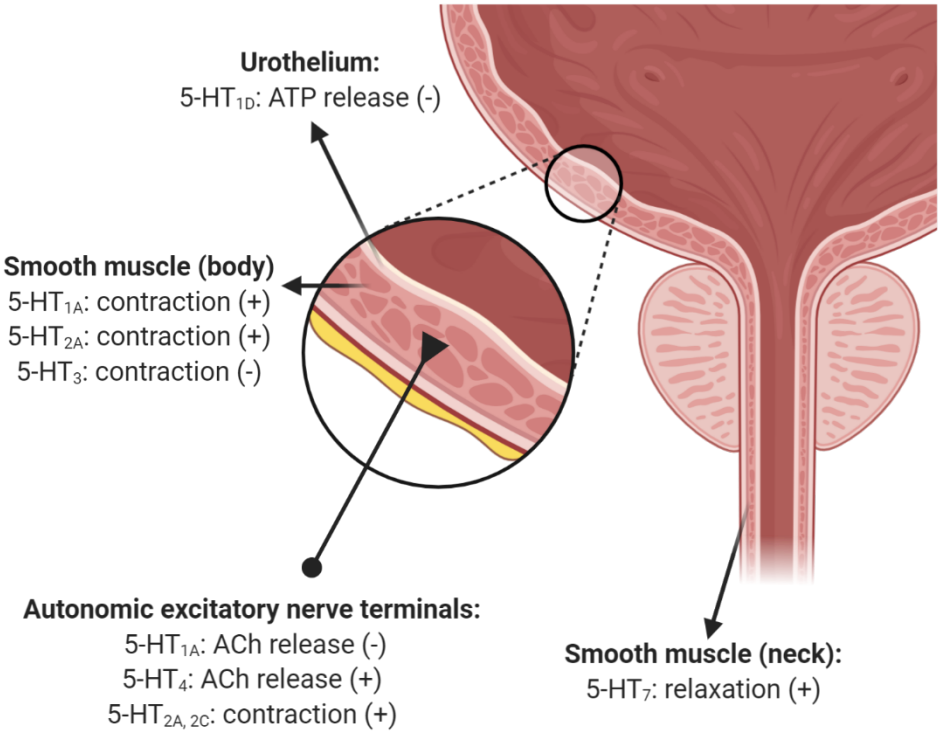

Figure 5.2: Functions of peripheral serotonin (5-HT) receptor subtypes in urinary bladder. + Induction or facilitation, - Inhibition. (Created with BioRender.com by the author).

While the location and general action of 5-HT receptors have been somewhat described in the literature, it remains unclear as to where 5-HT is being released. Due to the fact

that peripheral nerves do not release 5-HT when activated, it was suggested that platelets or mast cells may be releasing 5-HT (Yazaki et al., 1987; Yu et al., 1999).

In summary, 5-HT receptor subtypes on detrusor smooth muscle have been found to induce contraction or relaxation, while receptors on autonomic efferent terminals indirectly influence contraction by modifying purinergic or cholinergic transmission (Matsumoto-Miyai et al., 2015).

Sertraline

Sertraline (Zoloft) is a commonly prescribed SSRI, which was introduced as a treatment of depression in 1992 (Lewis et al., 2019). Sertraline is an orally ingested drug which reaches its maximum plasma concentration within 6-8 hours and the half-life is approximately 1 day (Vanharten, 1993).

Chronic stress alters neuronal circuits in the brain, leading to disruption of intracellular signalling and loss of function (Flak et al., 2012; Fontella et al., 2004). Sertraline has been widely documented to inhibit reuptake of serotonin into the presynaptic neuron, thereby increasing the neurotransmitter in the synaptic cleft (Hiemke & Hartter, 2000). While the blockage of serotonin reuptake is rapid, therapeutic action requires long term treatment due to the adaptive mechanisms of the presynaptic and postsynaptic neuron (Celada, Puig, Amargos-Bosch, Adell, & Artigas, 2004). A study of rhesus monkeys found that elevation of serotonin, after administration of sertraline, remains constant during prolonged administration, however, quickly returns to baseline after discontinuation of the drug (Anderson et al., 2005). While this study suggests rapid serotonin increase upon initial sertraline treatment, other studies have observed autoreceptor-mediated decreases in 5-HT neuronal firing which is believed to contribute to latency of sertraline response (Gray et al., 2013). There are alternative explanations for gradual onset,

including a downregulation of 5-HT transporters over the first 2-3 weeks of treatment. This results in reduced clearance of 5-HT from the synaptic cleft, leading to increased 5-HT in the extracellular fluid and increased receptor stimulation (Benmansour, Owens, Cecchi, Morilak, & Frazer, 2002). Clinical studies of sertraline reveal that depressive symptoms reduce within the first six weeks of treatment, however patients observed improvements in anxiety, quality of life and overall mental health before this time (Lewis et al., 2019).

Anxiolytics/anti-depressants and the Bladder

Overactive bladder (OAB) and other bladder pathologies are known to greatly hinder quality of life of sufferers. Due to the correlation between decreased quality of life and depression, clinical and experimental studies have been conducted which look at the effect of antidepressants on OAB, although the results are often conflicting. A clinical study conducted in the United States found that males taking certain antidepressants, including sertraline, experienced an increase in the incidence and symptom severity of OAB (Solmaz et al., 2017). Conflicting with this, a prospective trial was conducted to measure OAB symptoms in women taking antidepressants and found that sertraline treated patients had the lowest prevalence of symptoms (Albayrak et al., 2015). Some studies have observed that tricyclic antidepressants (TCAs) are effective in treating interstitial cystitis and some OAB symptoms, however, these drugs lack specificity for receptors of the bladder (Solmaz et al., 2017). Instead, a study by Hillelsohn, Rais-Bahrami, Bagadiya, Kashan, and Weiss (2013), has used a different TCA, desipramine, and found that it is a potential useful treatment in patients with OAB, especially those who also experience bladder pain.

Though the association between bladder symptoms and depression has been documented clinically, no studies have investigated if current clinical therapies such as the SSRI sertraline are of therapeutic benefit in managing the bladder dysfunction associated with psychological stress. However, one experimental study assessed the effects of two SSRIs, sertraline, and fluoxetine, on changes in bladder contractility caused by forced swim test (FST). While the FST induced detrusor hypercontractility, this effect was abolished by long-term treatment with both anti-depressants (Bilge et al., 2008). Another study used female rats and subjected them to corticosterone treatment and the forced swim test to measure levels of depression. The study found that this caused detrusor inflammation and overactivity as well as depression. Treatment with the SNRI, duloxetine improved detrusor overactivity and depression via central mechanisms (Wrobel et al., 2020).

The aforementioned clinical studies of sertraline in overactive bladder patients highlight the efficacy of the drug and the lack of adverse effects experienced by these patients. It is for this reason that sertraline was chosen to be used in the current study.

Aims

The aim of the present study was to investigate the effects of sertraline on bladder dysfunction caused by water avoidance stress.

Specific aims were;

- To investigate the effects of sertraline on voiding dysfunction caused by water avoidance stress
- To assess the changes mediated by sertraline on bladder physiology after water avoidance stress
- To investigate if water avoidance stress or sertraline alter animal behaviour using the open field test

METHODS

Animals

Young adult female C57BL/6J mice were obtained and housed as outlined in the general methodology chapter (Chapter 2). Mice were randomly allocated into one of three groups: (1) Control/Unstressed, (2) Stressed and (3) Stress + Sertraline, with n=6 animals included in each group.

Sertraline Treatment Protocol

As stated in the background section, sertraline is a widely used SSRI to treat depression, with the least evidence of associated incontinence clinically, so it was chosen for use in the current study.

Sertraline (hydrochloride) was obtained from Cayman Chemicals and formulated as an oral suspension by National Custom Compounding Pharmacy and later diluted in the drinking water. A drug free suspension was added to the drinking water of mice in the other groups to account for any potential differences in water consumption due to the presence of the oral suspension.

Most studies looking at the efficacy of sertraline have commenced treatment between 1 and 2 weeks before the beginning of the stress exposure (Renoir et al., 2012). For this reason, mice in the present study were treated with the oral sertraline suspension 10-days prior to stress exposure and throughout the 10-day water avoidance stress exposure period (Di Rosso, Sterle, Cremaschi, & Genaro, 2018). The vehicle control suspension was also administered to age-matched unstressed control, and stressed mice throughout this period. The typical adult human dose for sertraline is 50 mg/day and was used to calculate the equivalent dose in mice (10 mg/kg/day), based on a published dose conversion guide for humans to animals (Nair & Jacob, 2016). The final

concentration of sertraline in the drinking water was 50 μg/ml which was available 24 hours a day, 7 days a week.

Water Avoidance Stress Protocol

Water avoidance stress protocol was undertaken in the same manner as outlined in chapter 3.

Open Field Tests

The open field test is a very common measure of general locomotor activity in mice. The test is widely used due to its effectiveness in describing several components of rodent behaviour. The procedure is one that allows the study of different strains of mice and comparison between the effects of different pharmacological agents (Seibenhener & Wooten, 2015). The open field test box was constructed as described in previous studies. The 'outer zone' consists of 16 segments while the inner zone consists of 9 segments (Seibenhener & Wooten, 2015). An example of the layout is given below in **Figure 5.3**.

Many aspects of behaviour can be assessed from an open field test, and the three parameters measured in this study were line crossing, thigmotaxis and rearing (Burne, McGrath, Eyles, & Mackay-Sim, 2005). The amount of line crossing, or ambulatory distance, is one of the most important parameters of the open field test. Thigmotaxis is the tendency of the subject to remain close to the walls and tends to increase with anxiety, as mice have a natural tendency to remain close to walls and away from brighter light, and was assessed as the number of times the animal entered the "inner zone" (Gould, 2009). The third parameter measured was rearing. Rearing behaviour occurs when the animal stands on its hind paws in an upright position. According to several studies,

increased rearing occurs in accordance with increase anxiety (Borta & Schwarting, 2005).

Stress and stress + sertraline treated mice were placed in the open field test box for 5 minutes on the day before drug/placebo treatment began, the day before first stress exposure and on day 10 after the final stress exposure. A go-pro camera was attached to the box and recorded the mouse behaviour during the open field test. This was analysed at a later date assessing the number of lines crossed, number of inner zone entries and number of rearing's.

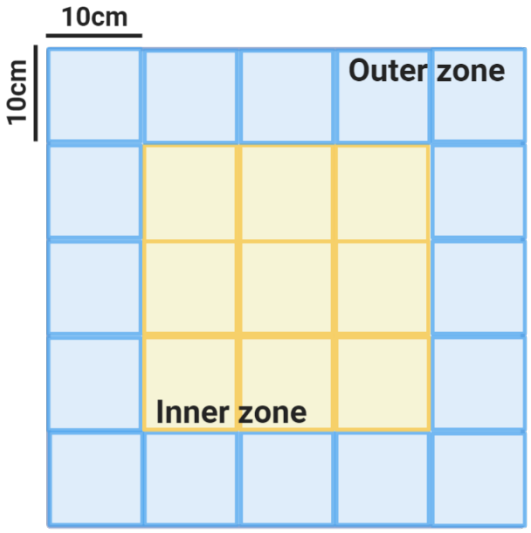

Figure 5.3: Example of layout of open field test box. Different zones are displayed. (Created with BioRender.com by the author).

Whole Bladder Preparation

Whole bladder preparation was performed as described in chapter 2, with the following differences due to the sex of the mice. When the abdominal region was secured to the dissection bath under the microscope, the female reproductive organs were removed including the uterus, fallopian tubes, ovaries and surrounding connective tissue. The whole bladder preparation was then continued as described in chapter 2.

Statistical Analysis

Two-way ANOVA with Tukey's post hoc test for multiple comparisons was used to compare the different time points, voiding variables and dose-response curves. Open field test data was analysed by a one-way ANOVA with multiple comparisons and Bonferroni tests. One-way ANOVA with Bonferroni post-hoc test was used to compare the difference between the three animal groups (Urothelial mediator release data, animal parameters, spontaneous activity data, response to ATP, αβmATP and KCl).

RESULTS

Animal Parameters and Voiding Behaviour

During the experimental protocol, water consumption and animal body weight were measured on day 0, 1, 3, 5, 7 and 10. This data was unchanged across treatment groups compared to the unstressed control and can be viewed in **TABLE 5.1**. After final experiments, bladders were weighed and weights were also unchanged across all groups.

TABLE 5.1: *Baseline body weight, water consumption and bladder weight (at day 10) in control (unstressed), water avoidance stress (stressed) and sertraline treated (stress + sert) mice (n=6).*

	Unstressed	Stressed	Stress + Sert
Body weight (g)	20.8 ± 0.12	21.8 ± 0.34	20.9 ± 0.23
Bladder weight (mg)	15.4 ± 1.05	17.8 ± 1.27	16.3 ± 0.12
Water consumption (g)	1.52 ± 0.18	1.66 ± 0.13	1.67 ± 0.11

A blood sample was taken at the time of euthanasia and plasma corticosterone levels were analysed. There was a significant increase in plasma corticosterone levels in the stressed group, 100.4 ± 24.09 μg/mL (n=6) compared to the unstressed group 33.00 ± 6.05 μg/mL (n=6) ($p = 0.03$). Sertraline treatment significantly decreased this, reducing levels of corticosterone to 23.0 ± 11.4 μg/mL (n=6) ($p = 0.01$), **(Figure 5.4)**.

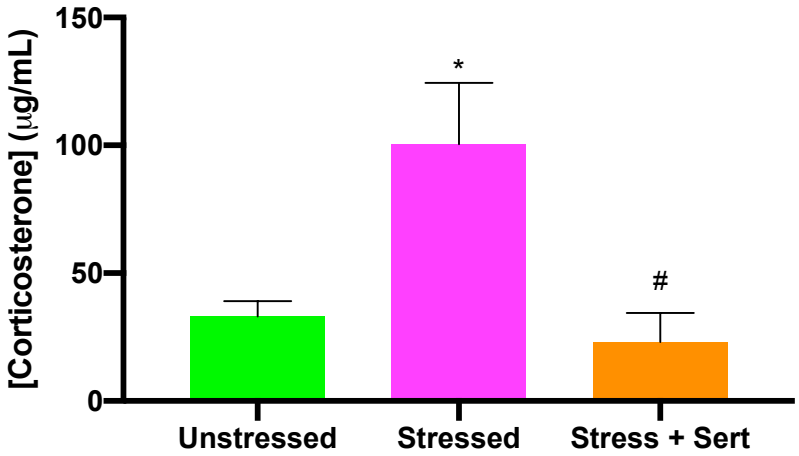

Figure 5.4: Plasma corticosterone levels in unstressed, stressed and sertraline treatment (stress + sert) mice. Datum is represented as mean ± SEM (n = 6). Analysis was performed as a one-way ANOVA with Tukey multiple comparisons test ($^{\#}p < 0.05$).

As reported in the previous chapter, water avoidance stress resulted in a significant increase in urinary frequency in the Stressed group when compared to unstressed control animals, as seen by the increase in voiding events **(Figure 5.5.A)**. This change was significant as early as day 1. The number of voiding events **(Figure 5.5.A)** was significantly reduced in the sertraline treated group compared to the stressed mice at day 3 onwards ($p < 0.001$), however, was not reduced back to unstressed levels and was significantly increased at day 3 ($p = 0.043$) and day 10 ($p = 0.014$) compared to unstressed mice. The average void size **(Figure 5.5.B)** was significantly decreased by stress but increased significantly in sertraline treated mice at day 0 ($p = 0.008$) and day 7 ($p = 0.014$) compared to the stressed group, with levels similar to unstressed animals. Total voided area **(Figure 5.5.C)** remained unchanged across all groups. The number of small voids **(Figure 5.5.D)** were significantly increased in the stressed group compared to the unstressed control at day 3 ($p = 0.008$) and day 5, 7 and 10 ($p < 0.001$), while sertraline treatment reduced the number of voids < 0.2 cm^2 significantly from day 5 onwards ($p < 0.001$) compared to the stressed group.

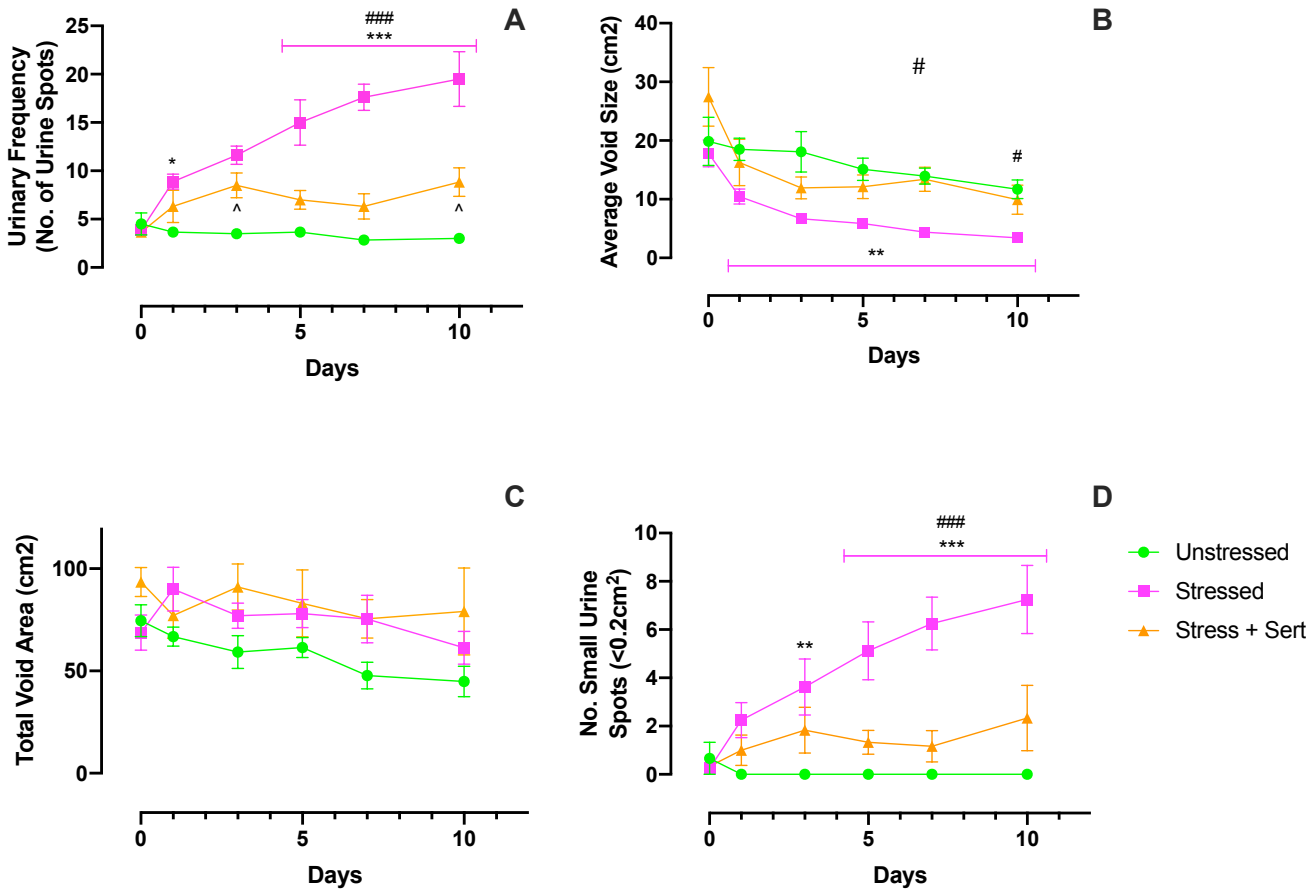

Figure 5.5: Voiding pattern analysis conducted in unstressed, stressed, and Sertraline treated (stress + sert) mice. (A) Urinary Frequency (number of voiding events), (B) Average void size, (C) Total voided area and (D) Number of small urine voids smaller than 0.2 cm². Datum is presented as mean ± SEM (n=6). Analysis was performed using two-way repeated measures ANOVA (** $p < 0.01$, *** $p < 0.001$, Unstressed vs. stressed) (# $p < 0.05$, ### $p < 0.001$, stressed vs. stress + sert).

Faecal pellets were counted **(Figure 5.6.A)** and weighed **(Figure 5.6.B)** after voiding pattern analysis. Results were variable across all groups and all days. There were some significant changes in the stressed group, compared to the unstressed group in both the number of faecal pellets and weight. Number of pellets at day 7, was significantly increased in the stressed group compared to the unstressed group ($p = 0.014$). Pellet weight was also significantly increased at day 1 ($p = 0.008$) and day 10 ($p = 0.033$).

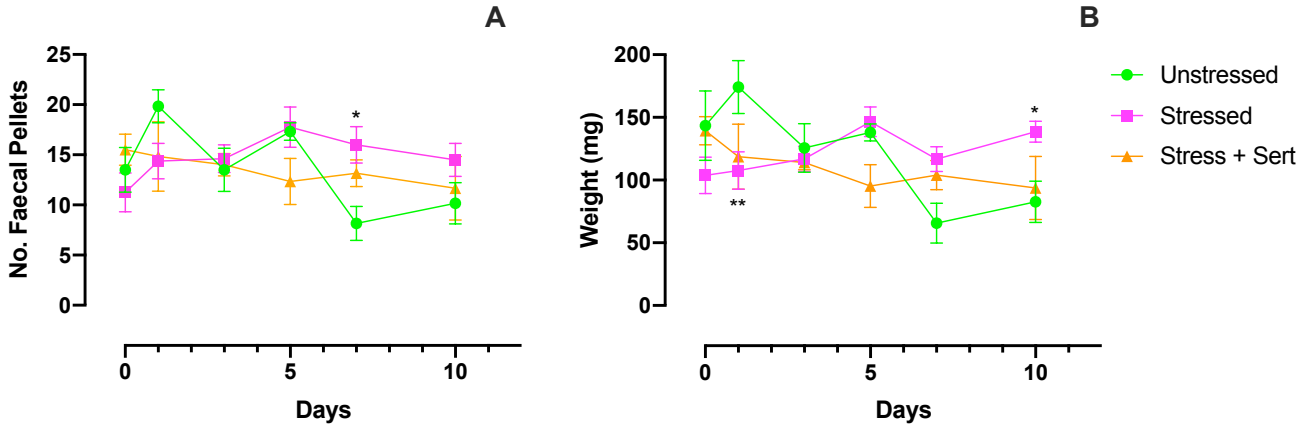

Figure 5.6: Faecal pellet analysis conducted in stressed, unstressed and Sertraline treated (stress + sert) mice. (A) Number of faecal pellets, (B) relative weight of faecal pellets. Datum is presented as mean ± SEM (n = 6). Analysis was performed using two-way repeated measures ANOVA (*$p < 0.05$, unstressed vs. stressed).

Three parameters of the open field test were measured in the sertraline treated group and stressed group. Line crossing, inner zone entries and number of rearing episodes were not altered in the stressed group **(Figure 5.7.A, C, E)**. Mice treated with sertraline entered the inner zone more frequently than the stressed group, however, this increase in activity was not significant **(Figure 5.7.D)**, with no alteration in the number of line crossings. Rearing behaviour in mice indicates stress and anxiety, and this was significantly decreased in the sertraline treated group at day 0 ($p < 0.036$) and day 10 ($p < 0.021$) **(Figure 5.7.F)**.

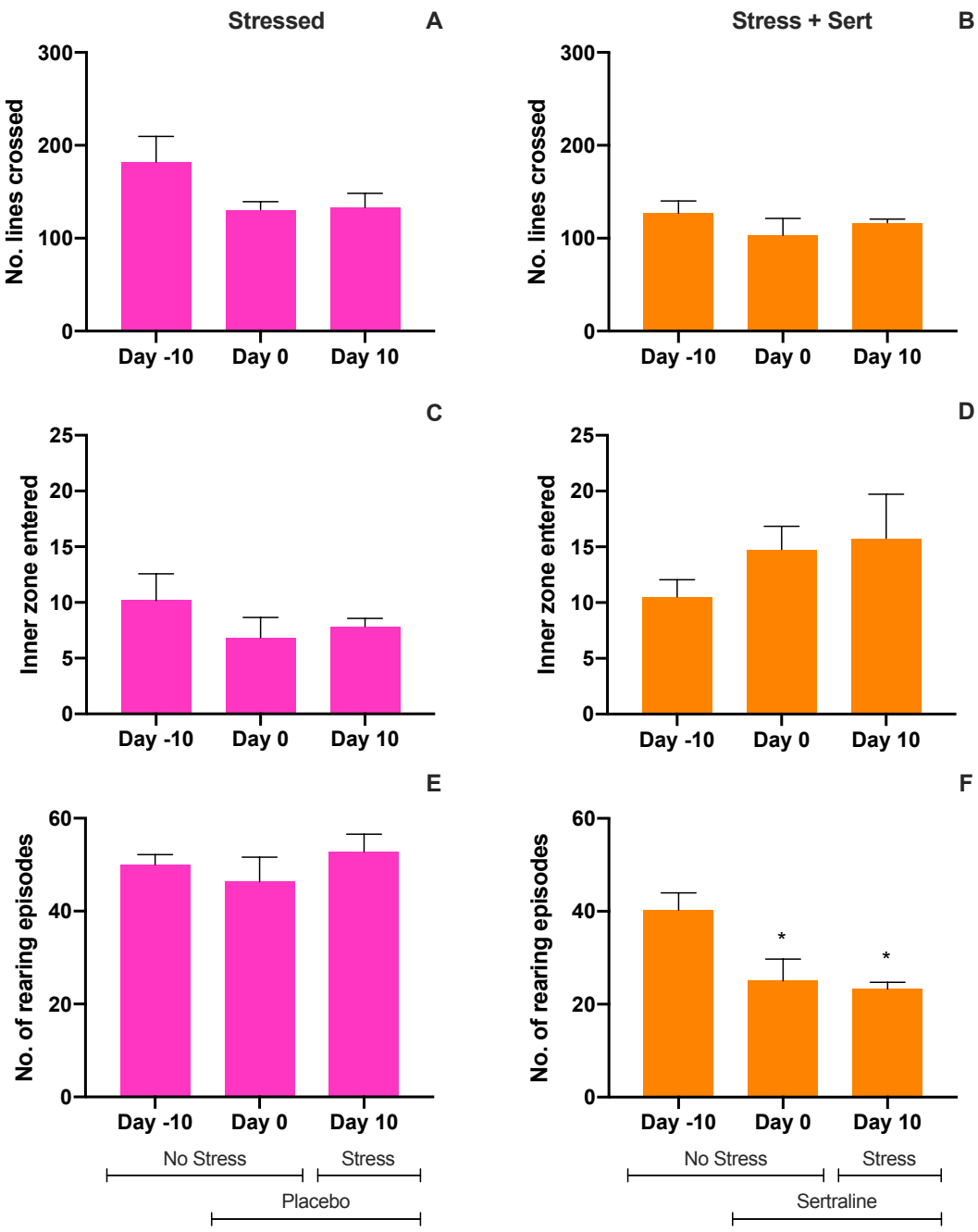

Figure 5.7: Open field test analysis conducted in stressed, unstressed and Sertraline treated (stress + sert) mice. (A & B) Number of lines crossed, (C & D) number of times the inner zone was entered and (E & F) number of rearing events. Datum is presented as mean ± SEM (n = 6). Analysis was performed as one-way ANOVA with Tukey multiple comparisons test (*$p < 0.05$).

Mediator Release

Intraluminal and serosal fluid was analysed for alterations in release of ATP and ACh. Volume at the time of collection was used to normalise mediator release in each sample and results are expressed as total ATP and ACh. Serosal and luminal ATP release **(Figure 5.8.A and B)** were unchanged across all groups. Total intraluminal ACh was not altered by stress or sertraline treatment **(Figure 5.8.C)**, however, total serosal ACh release was significantly increased in the stressed group compared to unstressed group (2.30 ± 0.15 nmols vs. 1.32 ± 0.18 nmol, $p = 0.006$, n=6) and significantly decreased in the sertraline treated group compared with the stressed group (1.55 ± 0.25 nmols, $p = 0.03$, n=6) **(Figure 5.8.D)**.

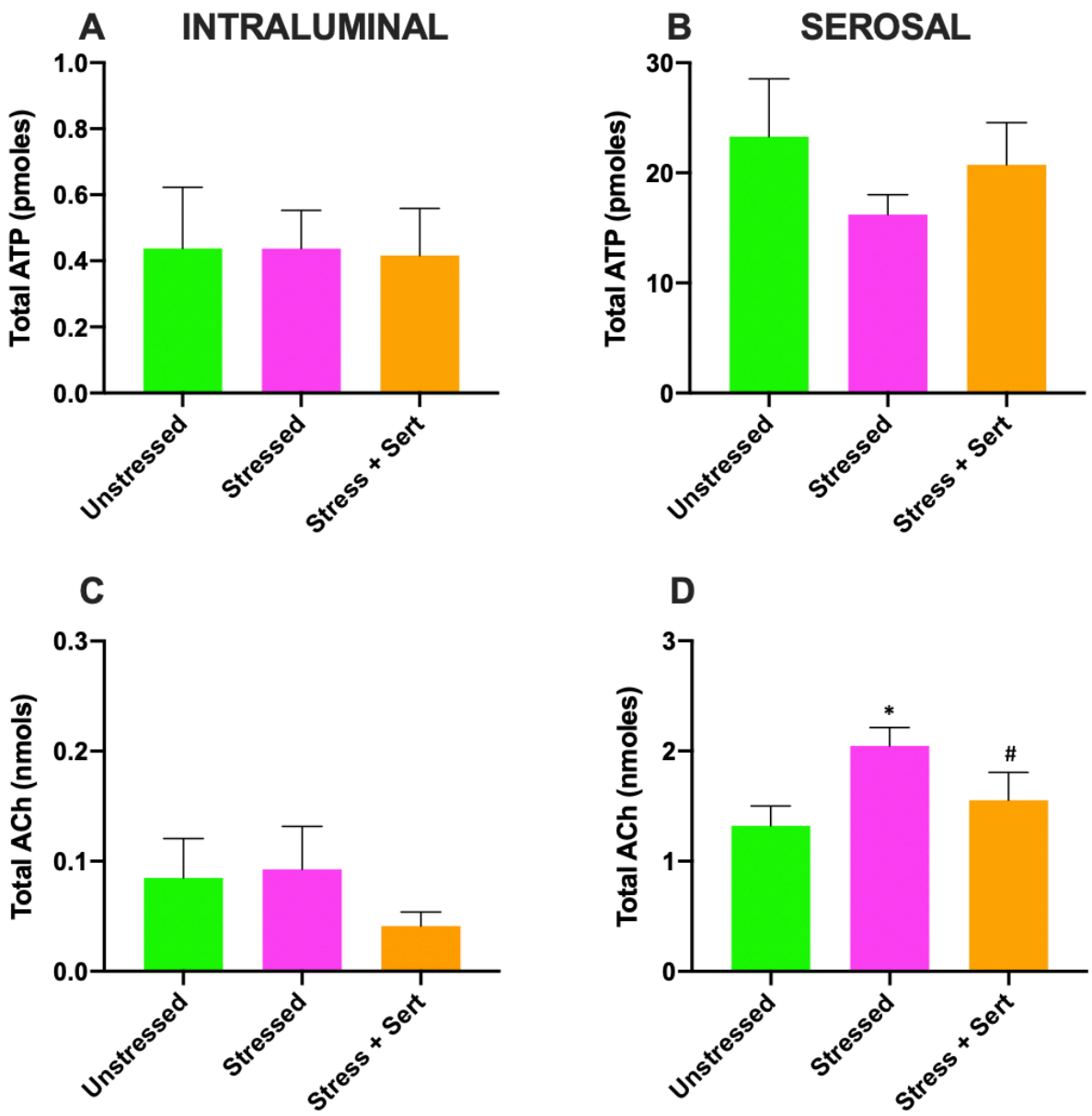

Figure 5.8: Total release of ATP and ACh into the (A & C) intraluminal and (B & D) serosal fluid collected following distensions of isolated bladders from stressed, unstressed and Sertraline treated (stress + sert) mice. Datum is represented as mean ± SEM (n = 6). Analysis was performed as a one-way ANOVA.

Bladder Compliance and Stretch-relaxation

A volume-pressure relationship was used to measure bladder compliance across the three groups. Bladder compliance was not significantly altered by stress and similarly sertraline treatment had no effect on compliance **(Figure 5.9)**.

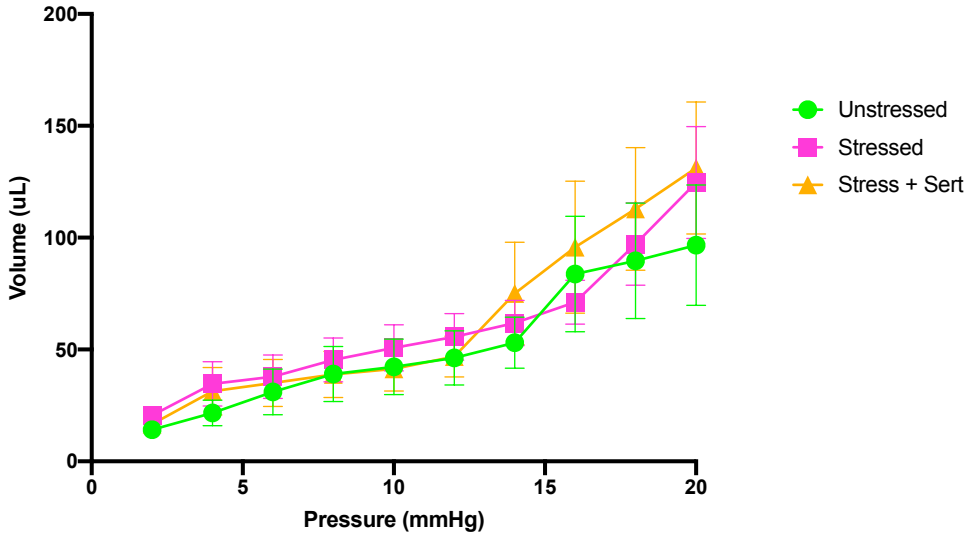

Figure 5.9: Volume-pressure relationship in isolated whole bladders stressed, unstressed and Sertraline treated (stress + sert) mice. Datum is represented as mean ± SEM (n = 6), analysed by two-way ANOVA.

After fluid collection, bladders were left to stabilise and equilibrate. Pressure stabilised over 30 minutes and the pressure-time relationship were assessed. There was a sharp decrease over the initial 8 minutes with a significant decrease in pressure in the stressed ($p = 0.01$) and sertraline treated ($p = 0.05$) groups compared to the unstressed group **(Figure 5.10)**. However, there was no significant difference in final pressure between the groups at the end of the stretch-relaxation period.

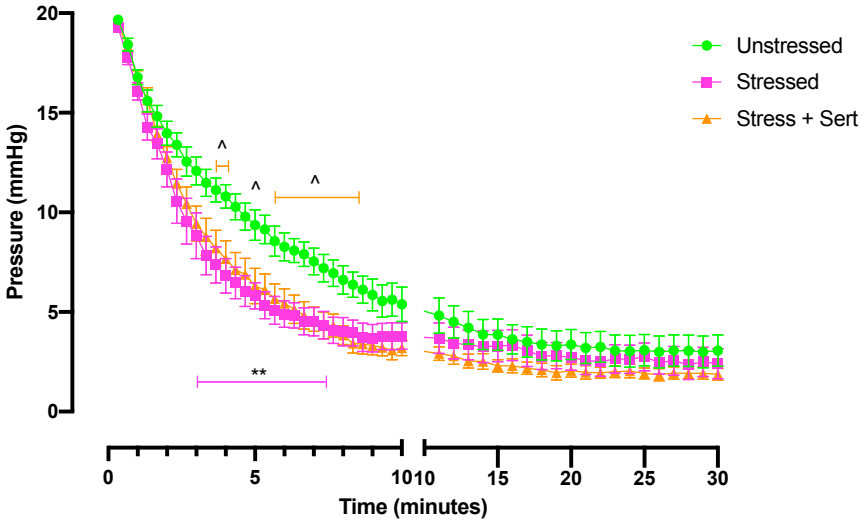

Figure 5.10: Pressure-time relationship for isolated bladders from stressed, unstressed and Sertraline treated (stress + sert) mice following distension to 20 mmHg. Datum is represented as mean ± SEM (n = 6), analysed by two-way ANOVA (**$p < 0.01$, Unstressed vs. stressed) (^$p < 0.05$, Unstressed vs. stress + sert).

Bladder Contractility and Electrical Field Stimulation

Response to KCl was measured at the end of each experiment to assess absolute contractile response from baseline in isolated bladders **(Figure 5.11).** Although the response in the isolated bladders from stressed mice was increased, there was no significant difference between all groups with responses of bladders from unstressed, stressed and sertraline treated mice recorded as 31.2 ± 3.27 mmHg, 38.2 ± 4.65 mmHg and 30.6 ± 4.43 mmHg, respectively, n=6.

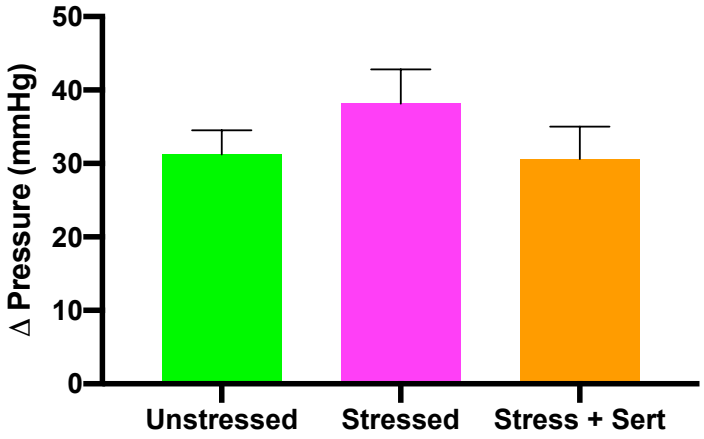

Figure 5.11: Pressure responses to KCl (60 mM) in isolated bladders from stressed, unstressed and Sertraline treated (stress + sert) mice. Datum is represented as mean ± SEM (n = 6) and analysed using an Ordinary one-way ANOVA with Tukey Analysis.

A full frequency-response curve to electrical field stimulation was performed at 1, 5, 10 and 20 Hz. An example of the frequency-response trace is shown in Chapter 2, General Methods, **Figure 2.5**. Stress did not change responses to electrical field stimulation, nor did treatment with the SSRI sertraline **(Figure 5.12.A)**. When the response to EFS was normalised to the KCl contraction **(Figure 5.12.B)**, the response in the stressed group was significantly depressed compared to unstressed controls at 10 Hz ($p = 0.008$) and 20 Hz ($p = 0.019$). The response in the stressed group at 10 Hz was also significantly decreased compared to the Stress + Sertraline group ($p = 0.014$).

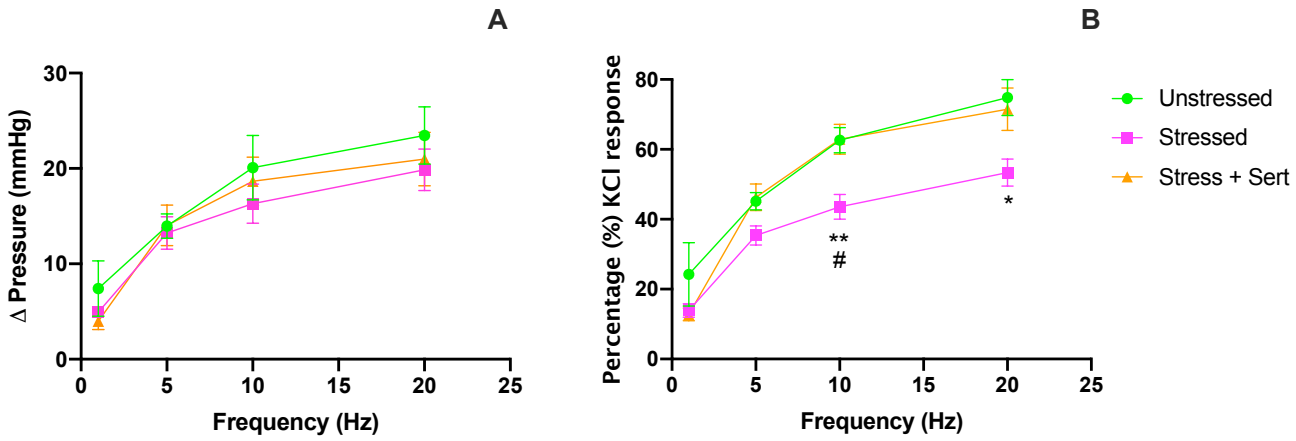

Figure 5.12: Response of isolated bladders to EFS, from stressed, unstressed and Sertraline treated (stress + sert) mice at 1, 5, 10 and 20 Hz. Responses were recorded as (A) a change in pressure from baseline and (B) a percentage of the KCl response. Datum is presented as the mean ± SEM (n = 6) and analysed using a two-way repeated measures ANOVA with Tukeys multiple comparison (*$p < 0.05$, **$p < 0.01$, Unstressed vs. stressed) (#$p < 0.01$, stress + sert vs. stressed).

Electrical field stimulation was repeated at 20 Hz in the absence and presence of several pharmacological agents. The addition of LNNA (100 μM) did not significantly alter bladder contraction to EFS, as seen in previous chapters, indicating that the inhibitory neurotransmitter NO was not involved in neurotransmission **(Figure 5.13.A)**. Addition of the muscarinic antagonist atropine (1 μM) reduced the response to EFS by 16.7 ± 3.03% (n=6) in unstressed control bladders. The same change was observed in the stressed and sertraline treated groups, 16.48 ± 2.84% and 13.62 ± 5.04%, respectively (n=6) **(Figure 5.13.B)**. Finally, with the addition of αβmATP (1 mM), to desensitise P_2X_1 purinoceptors, responses further decreased by 43.85 ± 5.36% (n=6) in unstressed controls, 51.15 ± 3.68% (n=6) in stressed and 52.63 ± 5.39% (n=6) in sertraline treated groups **(Figure 5.13.C)**. With the addition of TTX, responses were almost abolished in bladders from all groups **(Figure 5.13.A).** A two-way ANOVA with Tukey comparison revealed no significant difference in responses to pharmacological agents between groups.

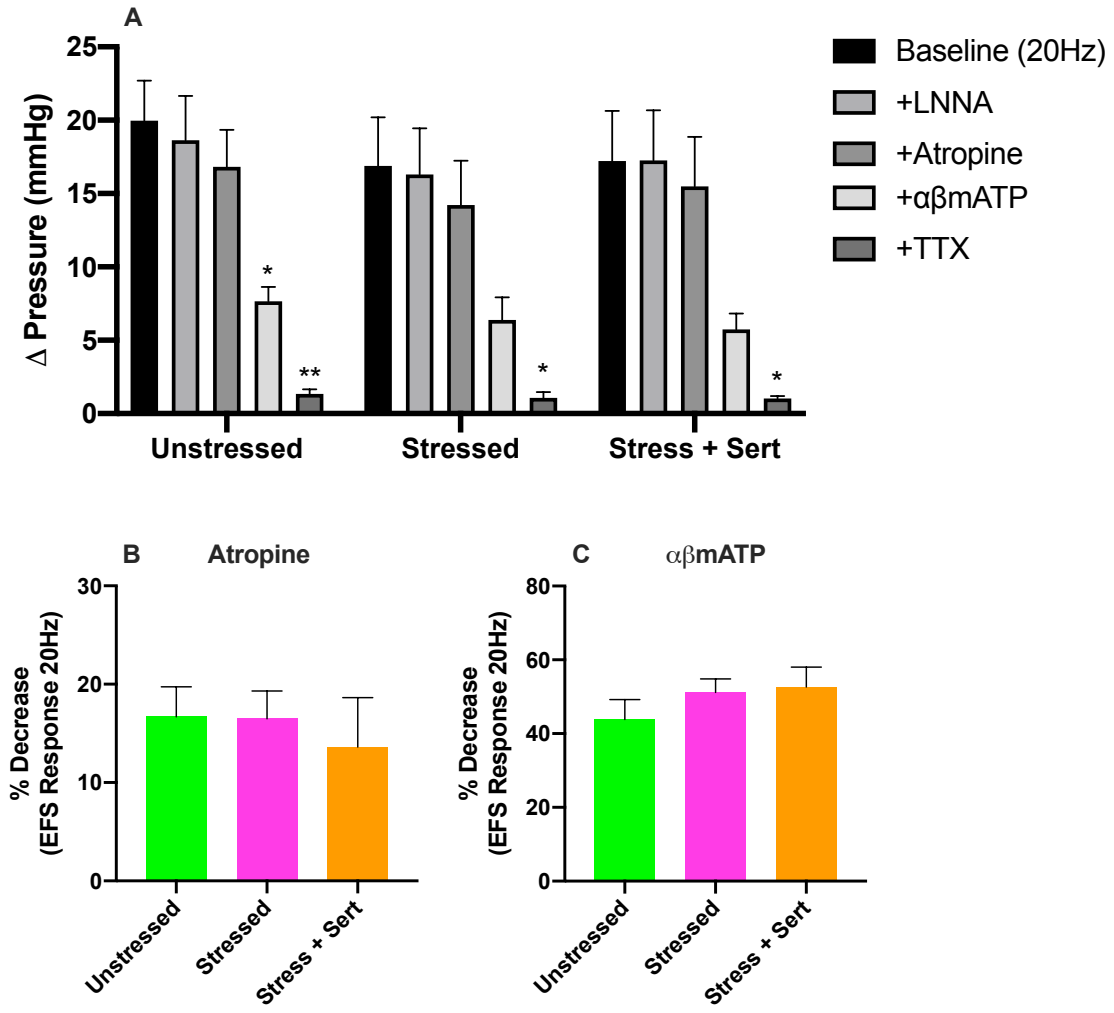

Figure 5.13: (A) Intravesical pressure responses to electrical field stimulation at 20Hz(baseline), and after addition of L-NNA (100 μM), atropine (1 μM), αβmATP (1 mM) and TTX (1 μM) to bladders from stressed, unstressed and Sertraline treated (stress + sert) mice. Percentage decrease in EFS response on addition of (B) atropine and (C) αβmATP to bladder from each group. Datum is expressed as mean ± SEM and analysed using two-way repeated measures ANOVA with Dunnett's multiple comparisons test for (A) and one-way ANOVA with Dunnett's multiple comparisons test for (B and C) (*$p < 0.05$, **$p < 0.01$ vs. Baseline (20 Hz)).

Response to Pharmacological Agents

Pharmacological agents were added to the bathing fluid and pressure responses measured. ATP (10 mM) and αβmATP (1 mM) were added to the bath to assess purinergic stimulation. In the stressed group the purinergic response of bladders to ATP was increased (11.3 ± 1.28 mmHg, n=6), although not significantly, compared to the unstressed group (8.89 ± 1.50 mmHg, n=6). Sertraline treatment did not significantly change this response (10.5 ± 1.26 mmHg, n=6) **(Figure 5.14.A)**. The response was normalised to KCl, with similar responses observed across the groups **(Figure 5.14B)**.

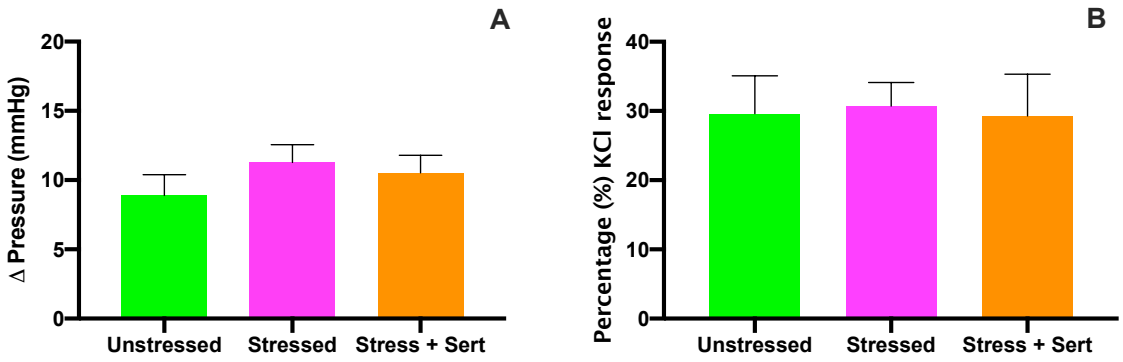

Figure 5.14: Pressure responses to (A) ATP (10 mM) and (B) ATP normalised to KCl response, in isolated whole bladders from stressed, unstressed and Sertraline treated (stress + sert) mice. Datum is represented as mean ± SEM (n = 6) and analysed using an ordinary one-way ANOVA with Tukeys comparison.

Contractile responses to αβmATP were assessed. Compared to unstressed controls, 17.43 ± 1.9 mmHg (n=6), there was no significant difference in response in bladders from the stressed, 15.23 ± 1.43 mmHg (n=6), and sertraline treated group, 16.92 ± 2.67 mmHg (n=6) **(Figure 5.15.A)**. As a percentage of the KCl response, there was no significant difference between the groups **(Figure 5.15.B)**.

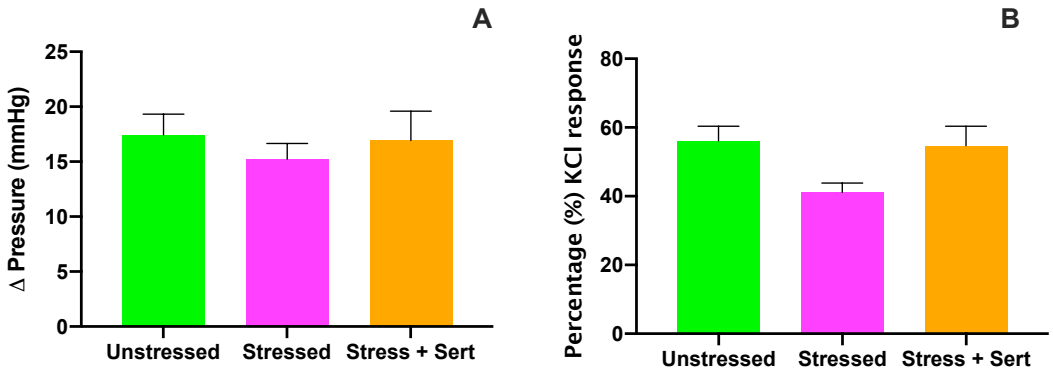

Figure 5.15: Pressure responses to (A) to $\alpha\beta$mATP (1 mM) and (B) as a percentage of the KCl contraction in bladders from stressed, unstressed and Sertraline treated (stress + sert) mice. Datum is represented as mean ± SEM (n = 6) and analysed using an ordinary one-way ANOVA with Tukey comparison.

The muscarinic agonist carbachol produced a concentration dependent increase in intravesical pressure in isolated bladders from unstressed, stressed and sertraline treated mice **(Figure 5.16.A)**. The maximum response was significantly enhanced in the stressed group compared to both the unstressed controls and sertraline treated animals **(Figure 5.16.A and Table 5.2)**. There was no significant difference in the pEC_{50} as highlighted in **Table 5.2**. The concentration-response curve was also assessed as a percentage of the KCl response, with no significant difference observed between the groups **(Figure 5.16.B)**. Sertraline treatment significantly reduced the increased contractile response in the stressed group, back to unstressed control levels **(Figure 5.16.A)**.

TABLE 5.2: *Whole bladder responses to carbachol in control (unstressed), water avoidance stress (stressed) and sertraline (stress + sert) treated mice (n=6).*

	Unstressed	Stressed	Stress + Sert
pEC_{50}	5.58 ± 0.03	5.40 ± 0.04	5.51 ± 0.07
Maximal response			
ΔPressure (mmHg)	43.47 ± 1.21	57.45 ± 3.36***	39.49 ± 4.21###
Response (%KCl)	145.20 ± 12.82	158.89 ± 12.92	136.02 ± 13.23

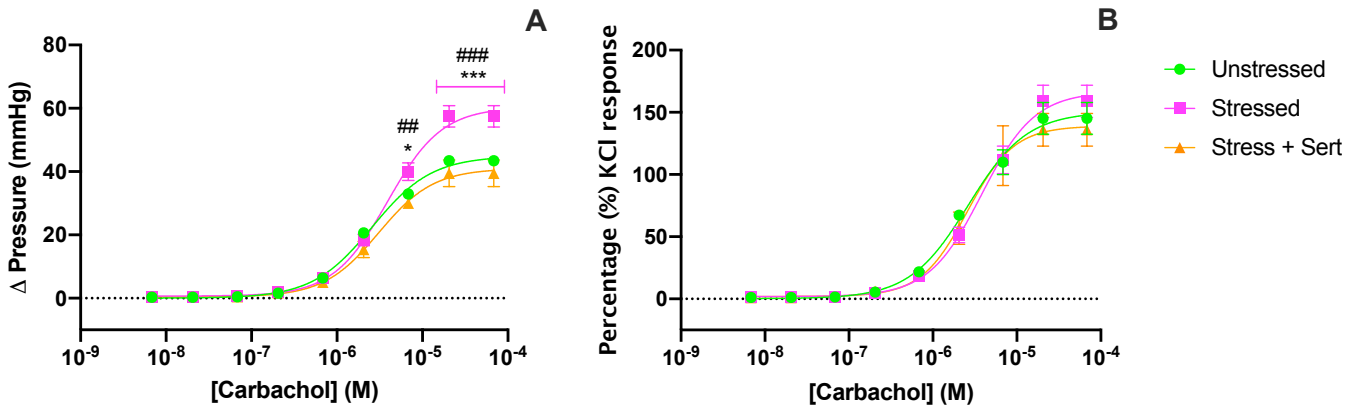

Figure 5.16: Carbachol concentration-response curves, from isolated whole bladder from stressed, unstressed and Sertraline treated (stress + sert) mice, recorded as (A) change in intravesical pressure from baseline and (B) change in intravesical pressure as a percentage of the KCl response. Datum is represented as mean ± SEM (n = 6), analysed using non-linear regression and two-way ANOVA with Tukey's multiple comparisons test (*$p < 0.05$, ***$p < 0.001$ Unstressed vs. stressed) (\#\#$p < 0.01$, \#\#\#$p < 0.001$ stress + sert vs. stressed).

A concentration-response to isoprenaline was performed to assess bladder relaxation following carbachol pre-contraction. There was no significant difference in the potency of isoprenaline as determined by pIC_{50} values, **(Table 5.3)** however, there was a significant decrease in the maximal relaxation response of bladders in the stress and sertraline treated groups compared to unstressed controls **(Figure 5.17.A and B).**

TABLE 5.3: Whole bladder responses to isoprenaline in control (unstressed), water avoidance stress (stressed) and sertraline treated (stress + sert) mice (n=6).

	Unstressed	Stressed	Stress + Sert
pIC_{50}	6.71 ± 0.10	6.83 ± 0.09	7.05 ± 0.08
Maximal response			
ΔPressure (mmHg)	- 5.91 ± 0.46	- 4.68 ± 0.31**	- 4.83 ± 0.31^
Response (% Decrease)	14.37 ± 10.61	34.09 ± 7.58	26.58 ± 7.06

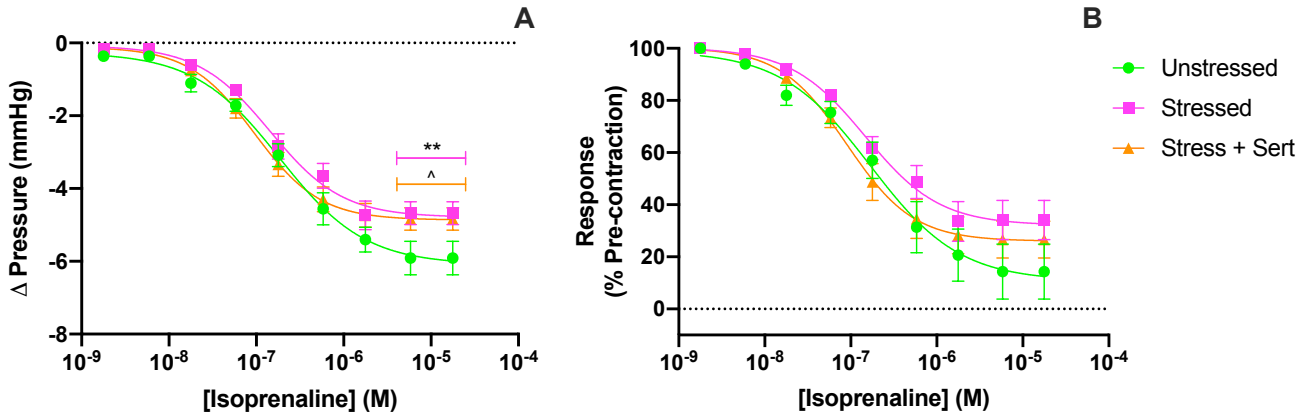

Figure 5.17: Effect of isoprenaline on isolated bladders, from stressed, unstressed and Sertraline treated (stress + sert) mice, with datum given as (A) raw data as change in pressure from pre-contraction, (B) change in intravesical pressure as a percentage of the KCl response. Datum represented as mean ± SEM (n = 6), using two-way ANOVA and non-linear regression (*$p < 0.05$, **$p < 0.01$, Unstressed vs. stressed) (^$p < 0.05$, unstressed vs. stress + sert).

Spontaneous Phasic Contractions

During the stretch-relaxation phase, the frequency of spontaneous phasic activity was increased in bladders from the stressed group compared to unstressed group, although not significantly **(Figure 5.18.A)**. However, sertraline treatment significantly decreased the frequency ($p = 0.032$) of spontaneous phasic contractions compared to the stressed group. The same trend was also observed in the amplitude of spontaneous activity **(Figure 5.18.B)**. Sertraline again significantly decreased the amplitude of spontaneous phasic contractions compared to the stressed group ($p = 0.006$).

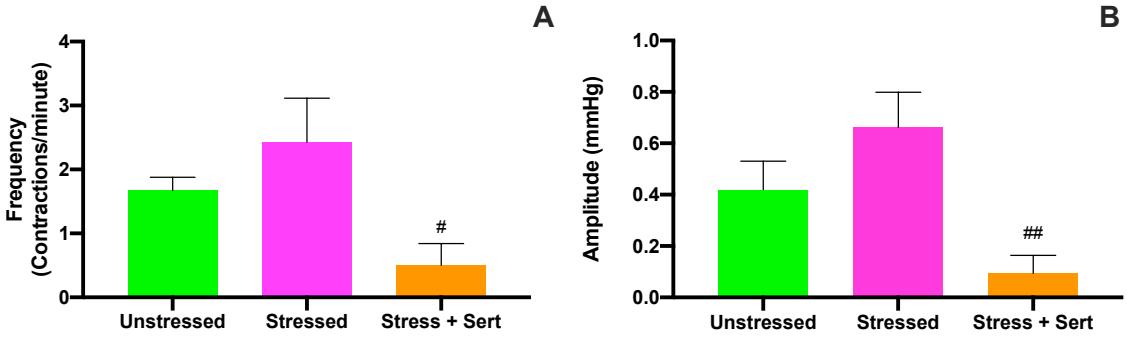

Figure 5.18: Spontaneous phasic contractions measured during the stretch-relaxation period as (A) frequency and (B) amplitude in isolated bladders from stressed, unstressed and Sertraline treated (stress + sert) mice. Datum is represented as mean ± SEM (n = 6), analysed using one-way ANOVA with Tukey's multiple comparison ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, stressed vs. stress + sert).

Phasic activity was also analysed after the tonic contraction to carbachol (1 µM). Frequency of phasic activity was significantly increased in the stressed group and sertraline treated group compared to the unstressed controls ($p < 0.001$ and $p = 0.032$, respectively) **(Figure 5.19.A)**. However, the frequency of contractions in bladders from sertraline treated mice was significantly lower compared to the stress only group ($p = 0.019$). The amplitude of phasic activity was unchanged across all groups **(Figure 5.19.B)**.

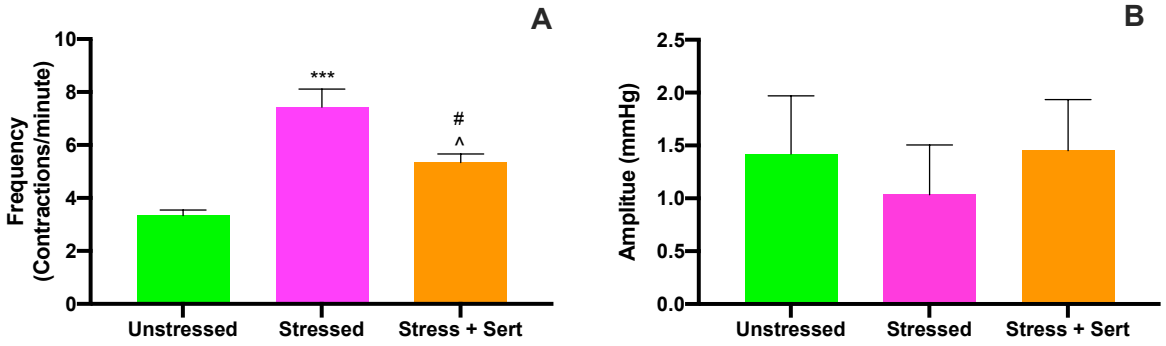

Figure 5.19: Tonic and phasic contractions after 1 µM carbachol in isolated whole bladders from stressed, unstressed and Sertraline treated (stress + sert) mice, following addition of carbachol (1 µM). (A) Frequency of phasic contractions and (B) Amplitude of phasic contractions. Datum is represented as mean ± SEM (n = 6), analysed using a one-way ANOVA (*$p < 0.001$, Unstressed vs. stressed) (#$p < 0.05$, stressed vs. stress + sert) (^$p < 0.05$, Unstressed vs. stress + sert).**

DISCUSSION

In previous chapters, water avoidance stress was shown to increase voiding frequency and cause changes in bladder physiology, including an increase in detrusor muscle contractility. SSRIs such as sertraline are commonly used in the clinical management of anxiety and depression, with a low side-effect profile compared to other anti-depressants, however their benefit in the management of stress-induced voiding dysfunction is unknown. Experimental studies have linked depletion of serotonin in the CNS to increases in urinary frequency and detrusor overactivity (Chiba et al., 2016), with serotonin depletion being postulated to play a role in the pathophysiology of OAB. This would suggest that an SSRI may be of therapeutic benefit in managing the voiding dysfunction associated with stress. This section will discuss the alterations in bladder physiology after sertraline treatment and examine the ability of sertraline to resolve the voiding changes caused by repeated water avoidance stress exposure.

Effects of Sertraline on Behavioural Responses in Open Field Test and Voiding Behaviour

The open field test was used to measure the anxious and depressive state of mice following water avoidance stress and sertraline treatment. Interestingly, the WAS model was not associated with anxiety like behaviour, with similar behaviours observed at baseline and following repeated stress. This supports a study by Hassan et al (2014), who similarly found 7-days of WAS in mice had little influence on behaviour (Hassan et al., 2014). Conversely, Bradesi et al. (2005) reported increased anxiety-like behaviours in male rats following 10-days of WAS exposure, and this may reflect a species or sex differences in response to WAS (Bradesi et al., 2005). Line crossing and therefore ambulatory distance was unchanged between the animal groups in the current study which means that the mice are not affected by inactivity or pain (Gould, 2009). Inner

zone entering was increased in the sertraline treated mice, although not significantly. This parameter measures the natural aversion rodents have to exposed areas, and because of the increased activity in the 'inner zone' by the sertraline treated mice, it can be concluded that these mice were suffering less from anxiety-like behaviour (Carola, D'Olimpio, Brunamonti, Mangia, & Renzi, 2002). Rearing was significantly decreased in the sertraline treated group compared to the stressed group, over the course of the stress period. There is still some debate as to what type of behaviour rearing indicates, however, the general consensus is that rearing is indicative of anxiety (Borta & Schwarting, 2005), therefore, sertraline treatment reduced anxiety-like behaviour which has been reported previously in rodents (Bikomo, Ebuehi, & Magbageola, 2017).

The primary outcome of this study was to determine whether treatment with sertraline would limit the impact of water avoidance stress on urinary frequency. Sertraline treatment significantly decreased voiding frequency over the course of the 10-days stress exposure compared to the untreated water avoidance stress group. While this change alludes to effective management of stress-induced voiding dysfunction, it must be noted that there are still days on which urinary frequency remained significantly elevated in the sertraline treated group compared to the unstressed controls. There have been no experimental or clinical studies which have investigated the effects of sertraline on urinary frequency with stress. However, from what we know of the actions of SSRIs like sertraline and the role of serotonergic mechanisms in regulating lower urinary tract function, the benefits of sertraline observed in the current study are not surprising.

Sertraline is an SSRI which increases the amount of available serotonin (Anderson et al., 2005), an important neurotransmitter which plays a role in the control of the lower urinary tract (Andersson & Pehrson, 2003). Serotonergic neurons are present in several regions of the CNS which are involved in providing descending inhibition to the bladder,

including the Onuf nuclei and the lumbosacral autonomic nuclei which innervate the urethral sphincters (Andersson & Pehrson, 2003). The raphe nuclei of the medulla has been shown to contain serotonergic neurons with descending projections to the bladder, urethra and external urethral sphincter (Espey, Du, & Downie, 1998).

Raymond et al. (2001) compiled a review discussing the 7 subtypes of serotonin receptors, and when serotonin is administered experimentally, it either results in inhibitory or facilitatory affects, depending on which receptor dominates. An experimental study on cats found that serotonin induces external urethral sphincter activity to prevent leakage of urine during the storage phase (Espey et al., 1998). This is also supported by other animal studies which have observed Onuf nucleus stimulation with serotonin to cause contraction of the external urethral sphincter (Thor, 2003). Experimental studies in cats suggest that stimulation of 5-HT receptors suppresses the processing of afferent input from the bladder and results in excitation of sympathetic and inhibition of parasympathetic preganglionic neurons (De Groat & Ryall, 1967; Espey, Downie, & Fine, 1992; Ryall & DeGroat, 1972).

Clinical studies have observed urinary hesitancy and retention after beginning treatment with sertraline (Lowenstein, Mueller, Sharma, & FitzGerald, 2007). As stated above, this may be due to the number of serotonergic neurons present within the neural centres of micturition. Increased control of the external urethral sphincter by the serotonergic neurons, may explain why the mice treated with sertraline showed decreased voiding frequency compared to the stressed group in the present study. Both clinical and experimental studies have shown that duloxetine, a noradrenaline-serotonin reuptake inhibitor, is of benefit in treatment of OAB (Steers et al., 2007; Wrobel, Rechberger, & Rechberger, 2018).

Effects of Sertraline on Corticosterone Release

The stress hormone corticosterone was increased by water avoidance stress, however, levels were significantly decreased following sertraline treatment. Many studies have linked stress and increased corticosterone within the blood stream, due to the hyperactivity of the HPA axis during stress (Keeney et al., 2006). One study has observed that chronic stress caused by the FST promotes anxiety and depression. The study treated the stressed mice with sertraline and found that the SSRI prevents the altered behaviour associated with increased corticosterone levels and stress (Ulloa et al., 2010). There is some evidence to indicate that synthesis of serotonin within the neuronal cells is regulated by substrate availability which is affected by L-tryptophan in the diet and cytosolic enzymes in the liver. When there is hyperactivity of the HPA axis, during stress, increased cortisol induces the breakdown of L-tryptophan, meaning that there is decreased substrate availability for the neuronal cells (Bano, Gitay, Ara, & Badawy, 2010). Clinically, there have been studies which have observed this decrease in serotonin with increased cortisol levels. One study of young women, observed that a number of SSRIs, including sertraline, reduce cortisol levels throughout the day (Ronaldson et al., 2018).

Effects of Sertraline on Mediator Release

As observed in previous chapters, serosal release of ACh was significantly increased in bladders from the stressed group. In addition to this, sertraline treatment significantly decreased serosal ACh release. It has been reported previously that ACh is released from the urothelium in a non-neuronal capacity (Yoshida et al., 2006) and along with ATP, has been linked to several bladder pathologies (Apodaca, Balestreire, & Birder, 2007). The urothelium is a structure that is believed to be an important component in sensory

function and neurotransmitter release and plays a role in the regulation of normal bladder function (Birder et al., 2010). Studies have shown that bladder afferent neurons express muscarinic receptor subtypes, M_2, M_3 and M_4, which suggests that urothelial ACh may play a role in regulating sensory processing (Nandigama et al., 2010). A study of bladder afferent activation has confirmed the involvement of the M_2 muscarinic receptors in rats with spinal cord injury (Matsumoto et al., 2012). Evidence also suggests that release of urothelial ACh may be involved in afferent function of the bladder (Daly et al., 2010). Greater ACh release in the stressed group during filling could therefore stimulate bladder sensory nerves, triggering the micturition reflex at lower volumes, which would explain the subsequent overactive bladder phenotype observed in the stressed animals.

Effects of Sertraline on Bladder Physiology

As seen in previous chapters, general bladder contractility was increased in mice in the stressed group. Although not significant, sertraline treatment reduced the contractile responses to KCl. There was however a significant decrease in maximal contraction to carbachol in bladders from the sertraline treated group, compared to the stressed group. There was also a significant decrease in frequency and amplitude of spontaneous phasic activity, and frequency of the phasic activity following tonic contractions to carbachol.

While the use of sertraline to treat psychological ailments has been well documented, little is known of the underlying effects the drug has on the bladder. As discussed previously, there has been some clinical studies which have investigated the urological side effects of sertraline, however, experimental studies have been limited. One study has used sertraline to treat detrusor hyperactivity induced by the FST. The study found that sertraline significantly decreased contractility to carbachol and potassium chloride. Due to the inhibition of KCl-stimulated contractile response by sertraline, the study

suggested that the mode of action of the SSRI was not via the muscarinic receptors themselves, but by the inhibition of downstream signalling or contractile mechanisms beyond the receptor (Bilge et al., 2008). The inhibition of contractile responses following sertraline treatment has been studied in a number of different tissues. A study looking at rat aortic rings found that calcium channels are inhibited by sertraline (Becker, Morel, Vanbellinghen, & Lebrun, 2004). Contractile responses to noradrenaline, KCl and EFS in rat isolated vas deferens was also inhibited by sertraline treatment via effects on calcium channels (Kalyoncu, Ozyavuz, & Karaoglu, 1999). KCl causes contraction of smooth muscle by opening calcium channels and increasing intracellular calcium levels, thereby inducing contractions that are independent of muscarinic or purinergic receptor activity (Bilge et al., 2008). The inhibitory action of sertraline on calcium channels may have contributed to the overall decrease in bladder contractility witnessed within the sertraline treated group in the present study. Due to the high number of 5-HT receptors present on the bladder, it is also possible that the increase of sertraline within the blood, following sertraline treatment, had a direct effect via these receptors. A study of pig urinary bladder neck found that 5-HT relaxed smooth muscle through 5-HT_7 receptors (Recio et al., 2009). This will be further discussed later in Chapter 7.

It is well established that detrusor smooth muscle exhibits spontaneous phasic activity (Levin et al., 1986) which is shown to be myogenic in origin (Buckner, Milicic, Daza, Coghlan, & Gopalakrishnan, 2002; Liu, Volfson, Horan, & Levin, 1998). Spontaneous activity in isolated detrusor muscle has been reported to be dependent on both calcium entry mechanisms and potassium channels and is modulated by the presence of the urothelium (Buckner et al., 2002; Vahabi, Sellers, Bijos, & Drake, 2013). Sertraline treatment had a significant effect on spontaneous phasic activity and phasic responses following the tonic contraction to the muscarinic agonist carbachol. After treatment with

sertraline, frequency of phasic activity was significantly decreased compared to the stressed group. While the anti-depressant effects of sertraline are typically attributed to inhibition of serotonin reuptake mechanisms, the biological actions of SSRIs are more complex with evidence of inhibition of sodium, potassium and calcium channels (Deak et al., 2000; Frizzo, 2017; Lee, Kim, Hyun, Park, & Kim, 2012; Ohno, Hibino, Lossin, Inanobe, & Kurachi, 2007). While it has previously been reported that sertraline has effects on general contractility, as discussed above, no studies have measured spontaneous activity after treatment with this drug. It may be postulated that due to the reported actions of sertraline on calcium and potassium channels, and the role of these channels in regulating the frequency and amplitude of phasic activity in the bladder; this may explain the decreased spontaneous activity observed in bladders from the sertraline treated group (Becker et al., 2004; Ohno et al., 2007). The therapeutic benefit of sertraline in improving voiding behaviour with stress exposure may in part be due to this reduction in phasic activity in the bladder.

CONCLUSION

Repeated exposure to water avoidance stress induced bladder overactivity and treatment with the anxiolytic, sertraline reduced the effects of stress. Voiding behaviour was decreased but did not return to control levels, whilst sertraline treatment abolished the stress-induced changes in bladder contractile activity. This chapter indicates that management of bladder dysfunction caused by psychological stress may be aided by the addition of sertraline.

CHAPTER 6: EFFECTS OF MIRABEGRON AND SOLIFENACIN ON BLADDER DYSFUNCTION

INTRODUCTION

Antimuscarinic agents are the most common oral pharmacotherapy available for treating OAB (Abrams et al., 2015). The antimuscarinic drugs main mode of action was originally thought to be via inhibition of detrusor smooth muscle contraction, however they also reduce sensory symptoms during the filling and storage phase of the micturition cycle (Batista et al., 2015). More recently, the β_3-adrenoceptor (β_3-AR) agonist, mirabegron was introduced and has shown similar efficacy to the antimuscarinics, but with a lower incidence of side effects (Andersson, 2017a). It is currently unclear whether these drugs are useful in managing voiding dysfunction caused by psychological stress. This study investigates the potential benefit of the muscarinic antagonist solifenacin and the β_3-AR agonist mirabegron in treating the increased urinary frequency observed with water avoidance stress.

Muscarinic Antagonist Solifenacin: Mechanism of Action

Micturition normally occurs following the release of Ach from parasympathetic nerves onto the muscarinic M_3 receptors of the detrusor muscle (Chilman-Blair & Bosch, 2004). This causes contraction of the smooth muscle to void stored urine (Andersson & Arner, 2004). Several antimuscarinic drugs have been developed which directly target muscarinic receptors and reduce symptoms of OAB and for many years these were the only available treatment. While these treatments are effective in treating symptoms **(Figure 6.1)**, they are associated with a number of side effects resulting in decreased persistence in patients (Duckett, Hall, & Woodward, 1998).

In the 1970s, the non-selective muscarinic antagonist oxybutynin became available, with clinical trials showing the drug was effective in managing the symptoms of OAB. However, oxybutynin is associated with numerous side-effects including dry mouth,

constipation and blurred vision (Riva & Casolati, 1984) leading to many patients discontinuing treatment (Sexton et al., 2011). Since the 1990's more selective muscarinic antagonists have been developed with the aim of improving efficacy and reducing side-effects.

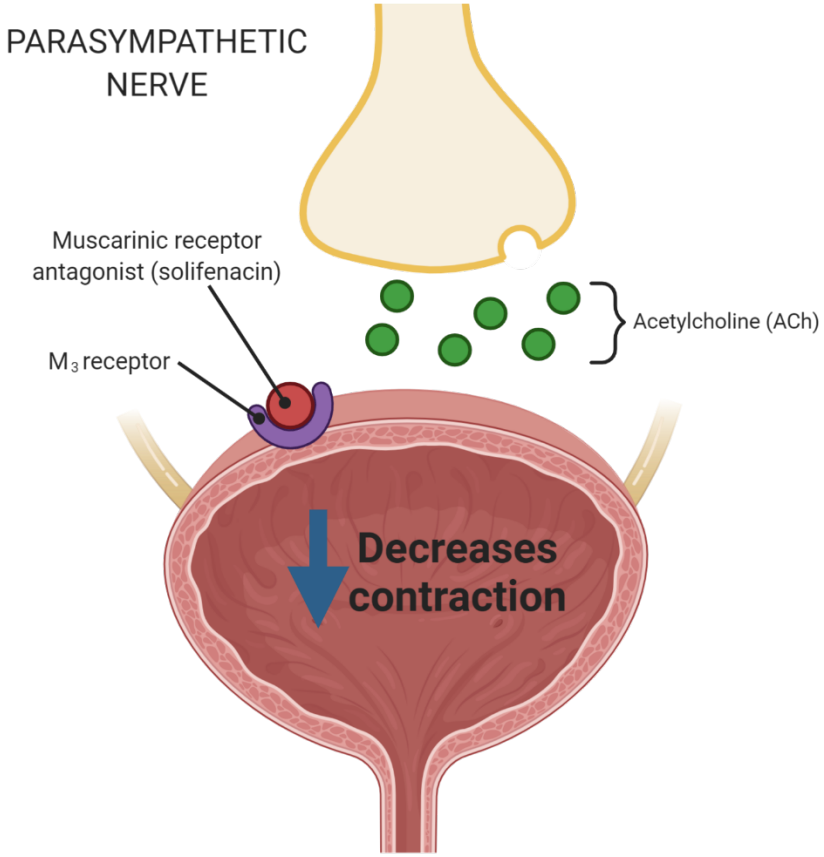

Figure 6.1: Diagram showing how solifenacin acts as a competitive muscarinic receptor (M_3) antagonist to decrease detrusor muscle contraction. Acetylcholine (ACh). (Created with BioRender.com by the author).

Solifenacin, a competitive M_3 selective receptor antagonist, was found to be more selective for bladder over salivary glands in *in vivo* and *in vitro* studies (Ohtake et al., 2004). Tolerability studies of solifenacin found that of 1637 patients, 81% completed 40 weeks of open label treatment where solifenacin was well tolerated and rates of side effects were low. In this study, only 4.7% of patients ceased treatment, and significant

improvements in OAB symptoms was observed after 52 weeks. Overall, tolerability of solifenacin was 85% and efficacy of solifenacin was 74% after long term treatment with solifenacin (Haab, Cardozo, Chapple, & Ridder, 2005).

There has been evidence that antimuscarinic agents targeting M_1 receptors cause cognitive impairments because of the way they cross the blood brain barrier (Kay et al., 2006). Studies of other M_3 receptor selective drugs, such as darifenacin and tolterodine have found that they do not cross the blood brain barrier to any significant extent to cause cognitive dysfunction (Kay et al., 2006). Solifenacin targets M_3 receptors, therefore cognitive dysfunction is unlikely.

There is no literature surrounding the use of OAB treatments in psychological stress-induced bladder dysfunction. Most studies have instead focussed on the quality-of-life related outcomes of patients with OAB symptoms being treated with solifenacin. One study performed two double-blind trials of 1033 and 857 patients to assess quality of life (QoL) outcomes after 12 weeks of treatment with solifenacin. The study found significant improvement in patient QoL after treatment with solifenacin compared to placebo treatment (Kelleher, Cardozo, Chapple, Haab, & Ridder, 2005). The VESIcare Efficacy and Research Study US has used a cohort of OAB patients who have described their bladder condition as severe. After treatment with solifenacin, the patients reported decreased urinary urgency and improvement of overall OAB symptoms as well as an improvement in health related QoL (Swift, Siami, & Forero-Schwanhaeuser, 2009).

Many studies of QoL improvement have focused on sleep disturbance as a symptom of OAB, also known as nocturia, the urge to urinate at night (Wein & Rovner, 2002). Sleep disturbance because of OAB symptoms has been reported by patients to interfere with cognitive function, impair concentration, increase anxiety and low self-esteem (Abrams, Kelleher, Kerr, & Rogers, 2000). A study of OAB and insomnia enrolled 15 patients into a

study looking at effects of solifenacin on both conditions. The study found that solifenacin significantly improved both OAB symptoms and insomnia scale results, improving both total sleep time and sleep efficacy (Takao et al., 2011).

β_3-Adrenoceptor Agonist Mirabegron: Mechanism of Action

The β_3-adrenoceptor (β_3-AR) is the most widely expressed adrenoceptor subtype on the detrusor muscle in humans (Michel, Ochodnicky, Homma, & Igawa, 2011). Sympathetic nerve activity triggers the release of NA onto the β_3-ARs, causing relaxation of the smooth muscle cells of the detrusor muscle and contraction of the urethra via α_1-adrenoceptors, thereby contributing to urine storage (Goulooze, Cohen, & Rissmann, 2015). Mirabegron is the first selective β_3-AR agonist and acts like noradrenaline to trigger detrusor muscle relaxation **(Figure 6.2)**, which is useful in the treatment of OAB syndrome (Andersson, 2017a). Mirabegron is one of the most selective OAB treatments and due to its success in clinical practice, there is increased interest in the mechanism of action of this agonist (Andersson, 2017b).

On a molecular level, mirabegron couples to G-proteins which increases intracellular cAMP, thereby activating potassium (K^+) channels and causing hyperpolarisation of detrusor smooth muscle (Igawa & Michel, 2013). There is also some evidence that mirabegron acts to inhibit spontaneous activity in the bladder by inhibiting micro-contractions which appear to be myogenic in origin (Aizawa, Homma, & Igawa, 2012). Studies have also shown that the β_3-AR agonist inhibits C- and Aδ-afferent nerves in the rat bladder (Aizawa, Igawa, Nishizawa, & Wyndaele, 2010). Both the C- and Aδ-afferent nerves convey sensations of bladder filling and a study looking at these fibres found that mirabegron inhibited afferent activities of both fibres (Aizawa et al., 2012), and this may contribute to its therapeutic benefit in patients with OAB.

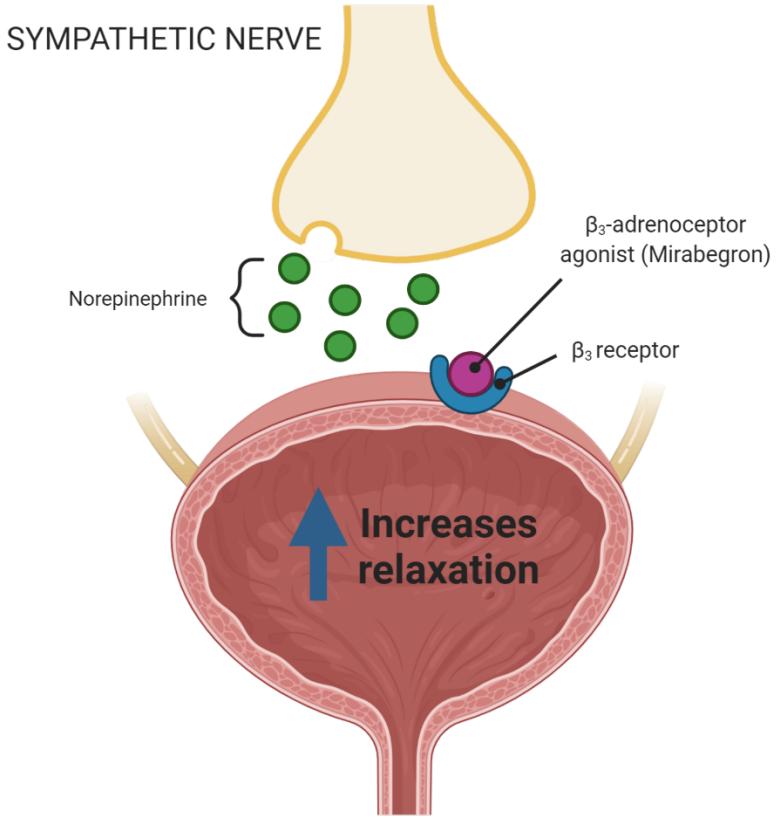

Figure 6.2: Diagram showing how mirabegron acts of β_3-adrenoceptors (β_3) to increase relaxation of the bladder smooth muscle (Created with BioRender.com by the author).

From the previous studies in this thesis, it is clear that psychological stress results in enhanced voiding and increased detrusor contractility, both phenotypic of overactive bladder syndrome. There have been no studies observing the effects of mirabegron on bladder dysfunction induced by psychological stress, although studies have focused on psychological stress as an outcome of overactive bladder.

One of the largest studies looking at the quality of life of OAB sufferers found that of 20,000 patients, 68% of the women and 60% of the men report bothersome symptoms which worsen scores of anxiety and depression in the Health Related Quality of Life (HRQoL) survey (Milsom, Kaplan, Coyne, Sexton, & Kopp, 2012). Another study has scored OAB, anxiety and depression and found that after treatment of OAB symptoms, there is also an improvement of anxiety symptoms (Kinjo, Yamaguchi, Tambo, Okegawa,

& Fukuhara, 2019). This correlates with other studies which found that female patients treated with mirabegron reported significantly decreased anxiety and depression on the Hospital Anxiety Depression Scale (HADS) and improved in QoL surveys (Athanasiou et al., 2020; Castro-Diaz et al., 2015). Another study looking at mirabegron treatment specifically used the European Quality of Life Survey (EuroQoL) to measure 5 dimensions, one of which was anxiety and depression. The study found that patients treated with mirabegron showed quicker and superior improvement in quality of life compared to other treatments (Pavesi et al., 2013). Persistence of OAB treatments has also been measured in a large prospective, non-interventional study. The study assessed QoL and found that patients receiving mirabegron treatment reported improvement in QoL and increased persistence rate of 53.8% for 12 months (Freeman et al., 2018).

Aims

Mirabegron and solifenacin are common clinical treatments for symptoms of overactive bladder. Due to the patient reported improvement in quality of life and decreased anxiety symptoms after treatment, the present study aims to observe the effects of mirabegron and solifenacin on water avoidance stress induced bladder dysfunction.

Specific aims were:

- To investigate the effects of solifenacin and mirabegron on voiding dysfunction caused by water avoidance stress.
- To assess how treatment with solifenacin or mirabegron affects bladder physiology in water avoidance stress mice

METHODS

Animals

Young adult female C57BL/6J mice were obtained and housed as outlined in general methodology chapter (Chapter 2). Mice were randomly allocated into one of four groups: (1) Control (Unstressed), (2) Stressed, (3) Stress + Mirabegron and (4) Stress + Solifenacin, with n=6 animals included in each group.

Drug protocol

Both mirabegron and solifenacin were obtained from Cayman Chemicals and National Custom Compounding Pharmacy (Merrimac, QLD) formulated both drugs into an oral suspension which was added to the animals drinking water. A placebo suspension was added to the drinking water of animals not in the drug treatment groups to control for variables in water consumption due to the presence of the oral suspension.

Several studies have observed and recorded the effects of oral mirabegron and solifenacin treatment on mice. Both drugs have been documented to take effect over a number of days and for this reason, mice in this protocol commenced treatment with oral mirabegron or solifenacin on the first day of the stress exposure period and continued for the remaining 10-days.

The typical adult human dose for mirabegron (10 mg/day) and solifenacin (50 mg/day) was used to calculate the equivalent does in mice (2 mg/kg/day for mirabegron and 10 mg/kg/day for solifenacin) based on a published dose conversion guide for humans to animals (Nair & Jacob, 2016). The final concentration of solifenacin in the drinking water was 50 μg/ml and for mirabegron 10 μg/ml which was available for consumption 24 hours per day, 7 days per week.

Water Avoidance Stress Protocol

Water avoidance stress protocol was performed in the same manner as outlined in chapter 3.

Whole Bladder Preparation

Whole bladder preparation was performed as described in chapter 2, with the following differences due to the sex of the mice. When the abdominal region was secured to the dissection bath under the microscope, the female reproductive organs were removed including the uterus, fallopian tubes, ovaries and surrounding connective tissue. The whole bladder preparation was then continued as described in chapter 2.

Statistical Analysis

Two-way ANOVA with Tukey's post hoc test for multiple comparisons was used to compare the different time point, voiding variables and dose-response curves. One-way ANOVA with Bonferroni post-hoc test was used to compare the difference between the four animal groups (urothelial mediator release data, animal parameters, spontaneous activity data, response to ATP, αβmATP and KCl).

RESULTS

Animal Parameters and Voiding

Animal body weight and water consumption were measured during the stress exposure period on days 0, 1, 3, 5, 7 and 10. The data was unchanged across all animal groups compared to the unstressed group, and data from day 10 is shown below in **Table 6.1.** Bladder weight was measured after whole bladder preparations and these parameters were also unchanged across all animal groups.

TABLE 6.1: *Baseline body weight, water consumption and bladder weight (at day 10) in control (unstressed), water avoidance stress (stressed), mirabegron (stress + mira) and solifenacin (stress + soli) treated mice (n=6).*

	Unstressed	Stressed	Stress + Mira	Stress + Soli
Body weight (g)	20.43 ± 0.28	21.35 ± 0.34	20.33 ± 0.48	20.03 ± 0.59
Bladder weight (mg)	17.21 ± 1.30	18.49 ± 1.00	16.88 ± 1.28	17.92 ± 2.36
Water consumption (g)	1.33 ± 0.16	1.47 ± 0.14	1.86 ± 0.09	1.97 ± 0.05

Blood samples were taken at the time of euthanasia and plasma corticosterone levels were measured. There was a significant increase in plasma corticosterone levels in the stressed group, 107± 21.90 μg/mL (n=6), compared to the unstressed group, 44.78 ± 8.89 μg/mL (p = 0.034) (n=6). Solifenacin significantly decreased plasma corticosterone levels, 37.01 ± 10.45 μg/mL (^p = 0.026) (n=6), and although not significantly different to the stressed group, mirabegron treatment also decreased corticosterone levels, 45.75 ± 17.03 μg/mL (p = 0.059) (n=6) **(Figure 6.3)**.

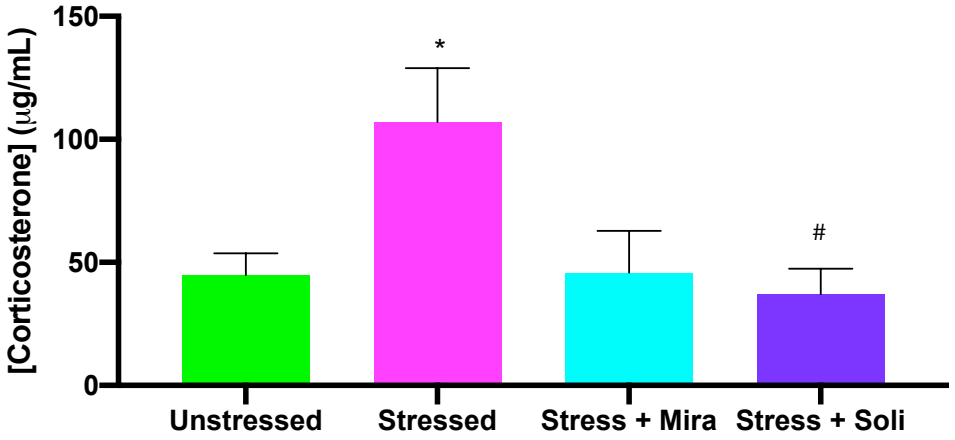

Figure 6.3: Plasma corticosterone levels in unstressed, stressed, mirabegron (Stress + Mira) or solifenacin (Stress + Soli) treated mice. Datum is represented as mean ± SEM (n = 6). Analysis was performed as a one-way ANOVA (*$p < 0.05$, Unstressed vs. stressed) (#$p < 0.05$, stressed vs. stress + soli).

As observed in the previous chapters, urinary frequency was significantly increased by WAS, which was associated with a significant decrease in the average void size and increase in the number of small spots **(Figure 6.4)**. Total voided area was unchanged by WAS. The number of voiding events **(Figure 6.4.A)** was significantly reduced in animals from both the mirabegron and solifenacin treated groups compared to the stressed mice from day 3 onwards. Average void size **(Figure 6.4.B)** was significantly decreased in the stressed group from day 1 onwards compared to the unstressed group. From day 3 onwards, mirabegron and solifenacin treatment significantly increased average void size to unstressed control levels. Total voided area remained unchanged across all groups, meaning that the mice urinated the same amount **(Figure 6.4.C)**. Number of small urine spots below < 0.2 cm^2 **(Figure 6.4.D)** was significantly increased in the stressed group compared to the unstressed group at day 3 and from day 5 onwards. Mirabegron and solifenacin significantly reduced the number of small voids to unstressed control levels.

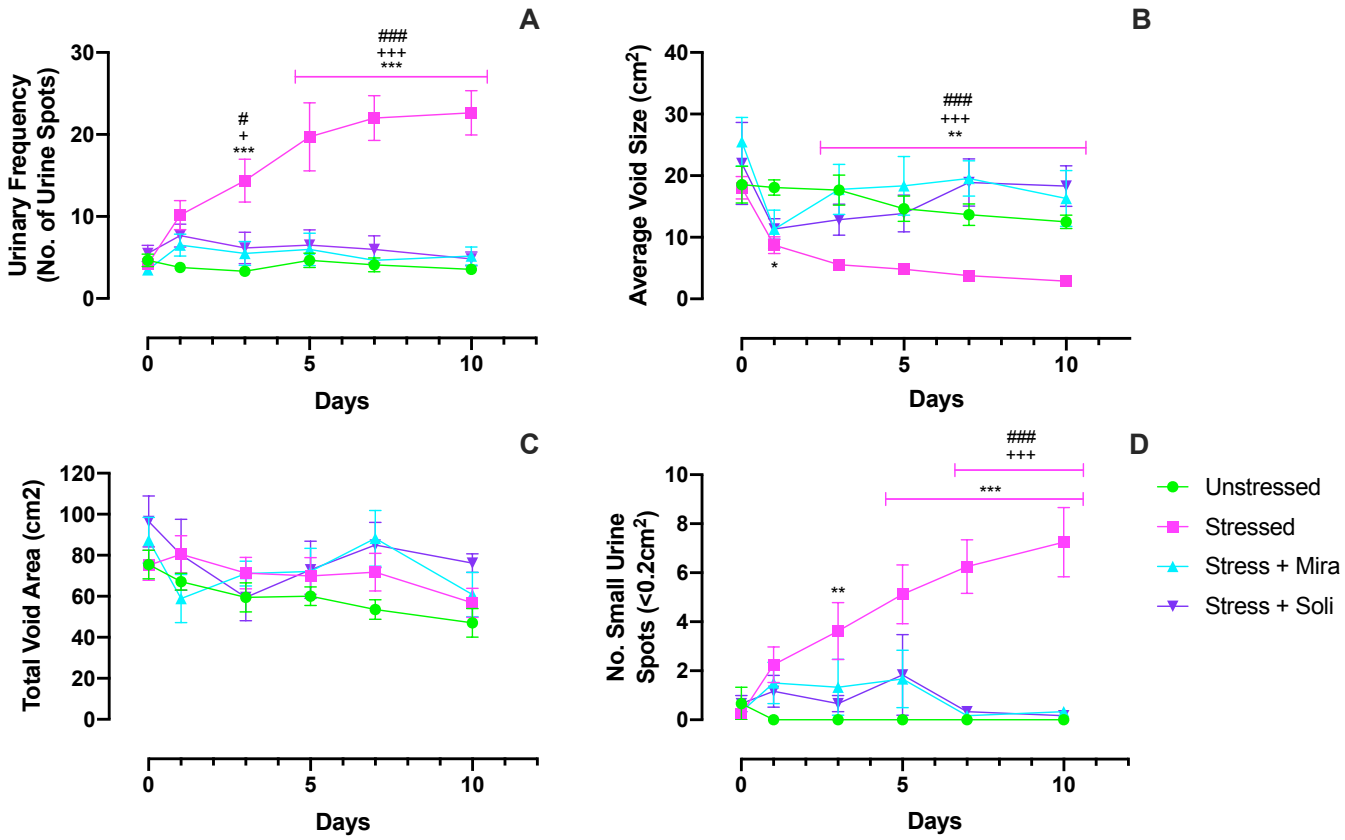

Figure 6.4: Voiding pattern analysis conducted in unstressed, stressed, mirabegron (Stress + Mira) or solifenacin (Stress + Soli) treated mice. (A) Number of voiding events, (B) Average voided area, (C) Total voided area and (D) Number of small urine voids smaller than 0.2 cm². Datum is presented as mean ± SEM (n = 9). Analysis was performed using two-way repeated measures ANOVA (**$p < 0.01$, ***$p < 0.001$, Unstressed vs. stressed) ($^{+}p < 0.05$, $^{+++}p < 0.001$, stressed vs. stress + mira) ($^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, stressed vs. stress + soli).

Faecal pellets were counted **(Figure 6.5.A)** and weighed **(Figure 6.5.B)** after voiding pattern analysis. Number of faecal pellets was variable and statistically unchanged across all groups. There were some significant changes in the weight of pellets at day 1 where in the stressed and mirabegron treated animals faecal weight was significantly decreased compared to the unstressed animals ($p = 0.0218$, $p = 0.0201$). At day 5, faecal weight was significantly decreased in the solifenacin treated group compared to the unstressed controls ($p = 0.0452$).

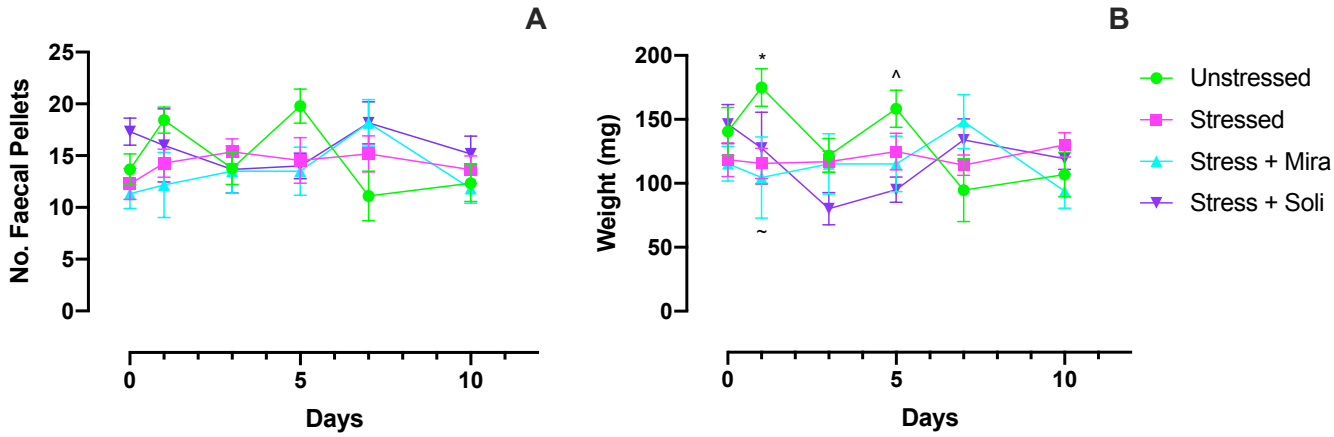

Figure 6.5: Faecal pellet analysis conducted in unstressed, stressed, mirabegron (Stress + Mira) or solifenacin (Stress + Soli) treated mice. (A) Number of faecal pellets, (B) relative weight of faecal pellets. Datum is presented as mean ± SEM (n = 9). Analysis was performed using two-way repeated measures ANOVA (*$p < 0.05$, unstressed vs. stressed) (~$p < 0.05$, unstressed vs. stress + mira) (^$p < 0.05$, unstressed vs. stress + soli).

Mediator Release

Serosal and intraluminal fluid was analysed for [ATP] and [ACh] content. The volume at time of fluid collection was used to normalise mediator concentration of each sample and results are expressed as total ATP and ACh. Both intraluminal ATP and ACh **(Figure 6.6.A and C)** concentrations were unchanged across all animal groups. Total serosal ATP was also unchanged across all groups **(Figure 6.6.B).** There was however a significant increase in total serosal ACh release in the stressed group (2.30 $\pm$ 0.15 nmols, n=6) compared to the unstressed controls (1.32 $\pm$ 0.18 nmols, n=6) ($p = 0.0005$). Total serosal ACh was then decreased significantly in the mirabegron treated group (1.48 $\pm$ 0.12 nmols, n=6) and solifenacin treated group (1.52 $\pm$ 0.11 nmols, n=6), compared to the stressed group ($p = 0.003$, $p = 0.007$).

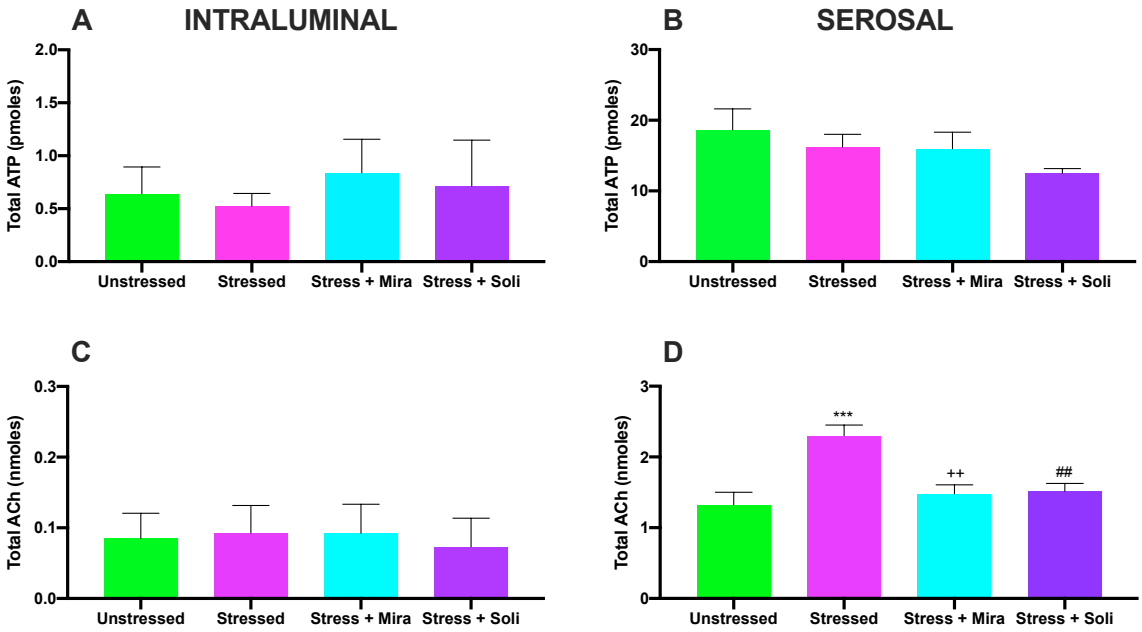

Figure 6.6: Total release of ATP and ACh into the (A & C) intraluminal and (B & D) serosal fluid collected following distensions of isolated bladders from unstressed, stressed, mirabegron (Stress + Mira) or solifenacin (Stress + Soli) treated mice. Datum is represented as mean ± SEM (n = 6). Analysis was performed as a one-way ANOVA (***$p < 0.001$, unstressed vs. stressed) ($^{++}p < 0.01$, stressed vs. stress + mira) ($^{\#\#}p < 0.01$, stressed vs. stress + soli).

Bladder Compliance and Stretch-relaxation

A volume-pressure relationship was used to measured bladder compliance across the four animal groups. Bladder compliance was not significantly altered by stress, mirabegron or solifenacin treatment **(Figure 6.7).**

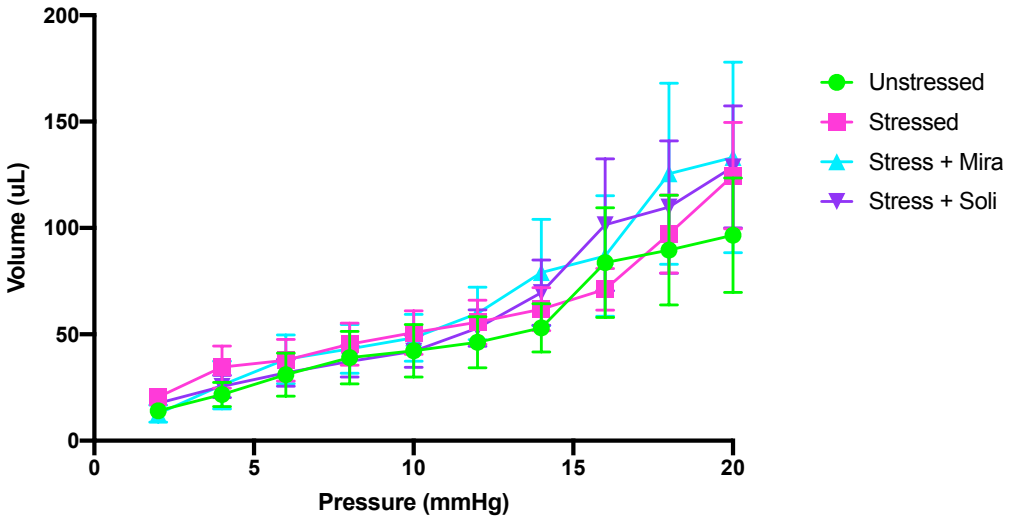

Figure 6.7: Volume pressure relationship for bladders from unstressed, stressed, mirabegron (Stress + Mira) or solifenacin (Stress + Soli) treated mice. Datum is represented as mean ± SEM (n = 9), analysed by two-way ANOVA.

After intraluminal and serosal fluid collection, bladders were left to equilibrate for 30 minutes. Over this period, the pressure-time relationship was assessed. Initially there was a sharp decrease over the first 8 minutes. There was no significant difference between the groups during the stretch-relaxation period or at final basal pressure **(Figure 6.8)**.

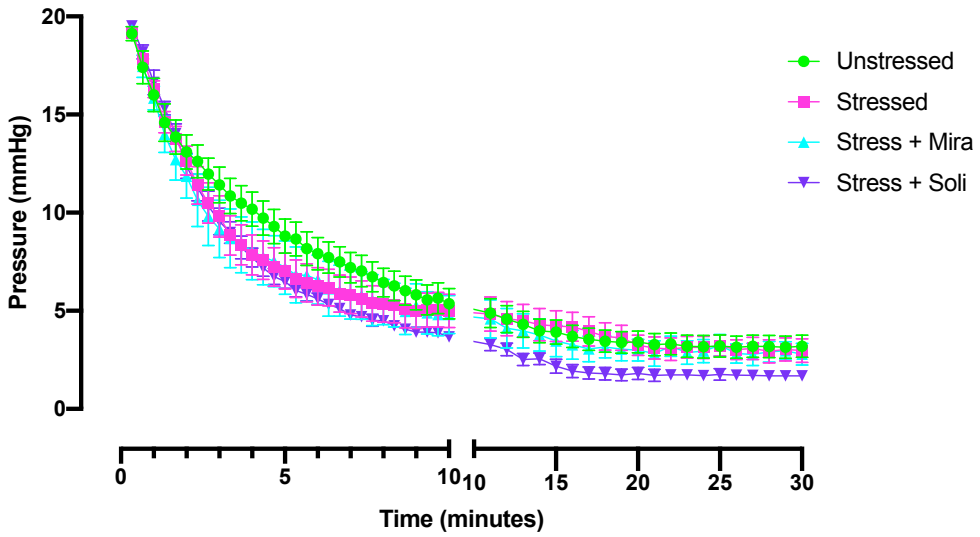

Figure 6.8: Pressure time relationship for isolated bladders from in unstressed, stressed, mirabegron (Stress + Mira) or solifenacin (Stress + Soli) treated mice following distension to 20 mmHg. Datum is represented as mean ± SEM (n = 9), analysed by two-way ANOVA.

Bladder Contractility and Electrical Field Stimulation

KCl responses were measured at the end of each experiment to assess non-receptor mediated bladder contractility **(Figure 6.9)**. There was no significant difference in KCl contractility between all groups with bladder responses from unstressed, stressed, mirabegron and solifenacin treated animals recorded as 31.23 ± 3.27 mmHg, 38.16 ± 4.65 mmHg, 36.92 ± 8.785 mmHg and 38.45 ± 5.277 mmHg, respectively (n=9).

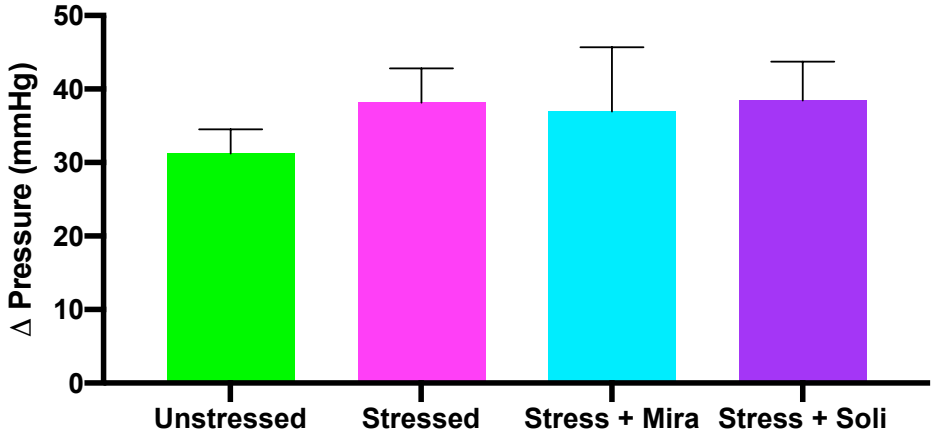

Figure 6.9: Pressure responses to KCl (60 mM) in isolated whole bladders in unstressed, stressed, mirabegron (Stress + Mira) or solifenacin (Stress + Soli) treated mice. Datum is represented as mean ± SEM (n = 9) and analysed using an Ordinary one-way ANOVA with Tukey Analysis.

Nerve mediated responses were assessed using electrical field stimulation at 1, 5, 10 and 20 Hz. An example of a frequency-response trace is shown in Chapter 2, **Figure 2.5**. Stress did not change the response to electrical field stimulation **(Figure 6.10.A)** and mirabegron and solifenacin did not have any effect on nerve mediated contractions. When the electrical field stimulation was normalised to KCl contraction **(Figure 6.10.B)**, the response in the stress groups was decreased compared to unstressed controls, at higher frequencies, however, this was not statistically significant.

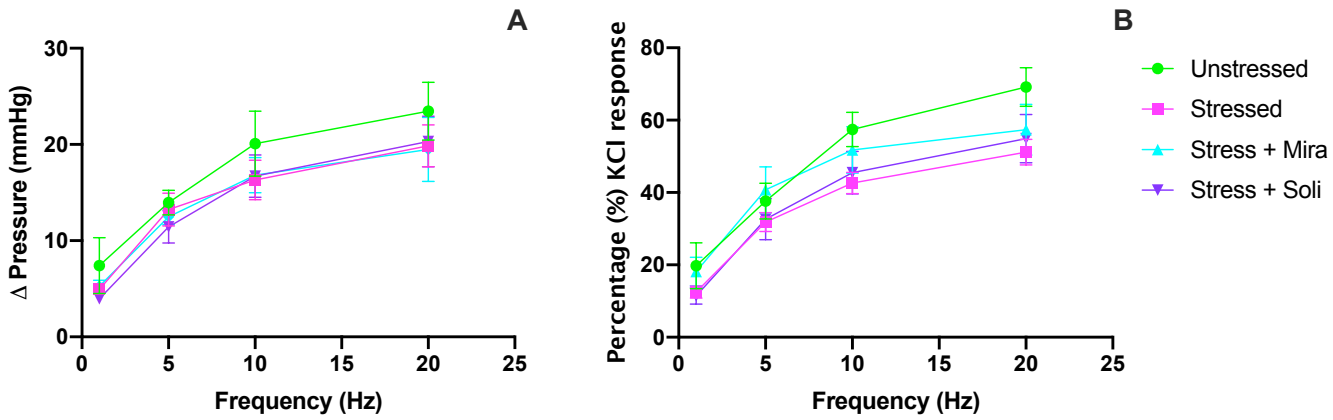

Figure 6.10: Responses of isolated whole bladders to electrical field stimulation from unstressed, stressed, mirabegron (Stress + Mira) or solifenacin (Stress + Soli) treated mice, at 1, 5, 10 and 20 Hz. Responses were recorded as (A) a change in pressure from baseline and (B) a percentage of the KCl response. Datum is presented as the mean ± SEM (n = 9) and analysed using a two-way repeated measures ANOVA with Tukeys multiple comparison.

Relative contribution of NO, ACh and ATP to nerve mediated responses was assessed using electrical field stimulation at 20Hz, with addition of pharmacological agents. The addition of LNNA did not significantly alter bladder contractions to EFS, as seen in previous chapters which indicates that the inhibitory neurotransmitter NO was not involved in neurotransmission **(Figure 6.11.A)**. Addition of the muscarinic antagonist atropine (1 μM) reduced the response to EFS by 16.85 ± 2.57% (n=9) in unstressed controls. There was no significant difference between the groups with the same change observed in the stressed (15.38 ± 2.69%, n=9), mirabegron treated (9.59 ± 1.73%, n=9) and solifenacin treated (14.91 ± 2.41%, n=9) groups **(Figure 6.11.B)**. With the addition of αβmATP to desensitise the P_2X_1 purinoceptors, responses further decreased by 43.83 ± 4.53% (n=9) in unstressed controls, 51.48 ± 3.21% (n=9) in stressed, 46.36 ± 6.03% (n=9) in mirabegron treated and 50.68 ± 8.18% (n=9) in solifenacin treated groups **(Figure 6.11.C)**. A two-way ANOVA with Tukey multiple comparisons revealed no significant difference in responses to pharmacological agents between groups.

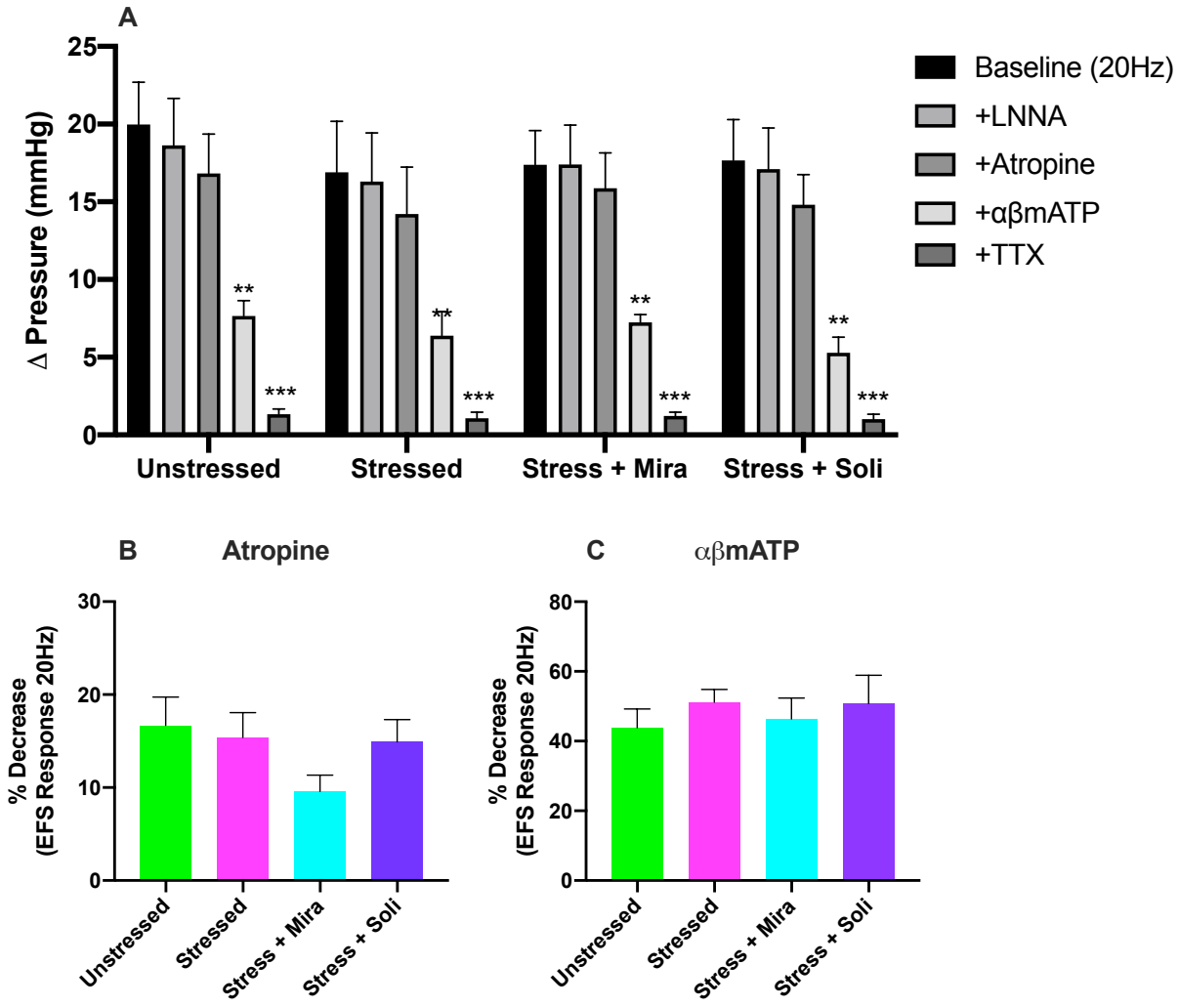

Figure 6.11: (A) Intravesical pressure responses to electrical field stimulation at 20 Hz (baseline), and after addition of L-NNA (100 μM), atropine (1 μM), αβmATP (1 mM) and TTX (1 μM) to bladders from unstressed, stressed, mirabegron (Stress + Mira) or solifenacin (Stress + Soli) treated mice. Percentage decrease in EFS response on addition of (B) atropine and (C) αβmATP to bladder from each group. Datum is expressed as mean ± SEM and analysed using two-way repeated measures ANOVA with Dunnett's multiple comparisons test for (A) and one-way ANOVA with Dunnett's multiple comparisons test for (B and C) (*vs Baseline (20 Hz)). Data analysed using two-way repeated measures ANOVA (**$p < 0.01$, ***$p < 0.001$ vs. Baseline (20 Hz)).

Response to Pharmacological Agents

ATP (10 mM) and αβmATP (1 mM) were added to the bath to assess purinergic stimulation. While not significant, ATP-induced pressure responses were increased slightly in bladders from the stressed group (11.28 ± 1.28 mmHg, n=9) compared to the unstressed control group (8.89 ± 1.51 mmHg, n=9). Mirabegron did not decrease the contractile response (11.2 ± 0.907 mmHg, n=9), however, solifenacin treatment did decrease ATP contractile responses by 8.96 ± 1.375 mmHg (n=9), although not significantly **(Figure 6.12.A)**. The response was normalised to the KCl response with no significant change indicated **(Figure 6.12.B)**.

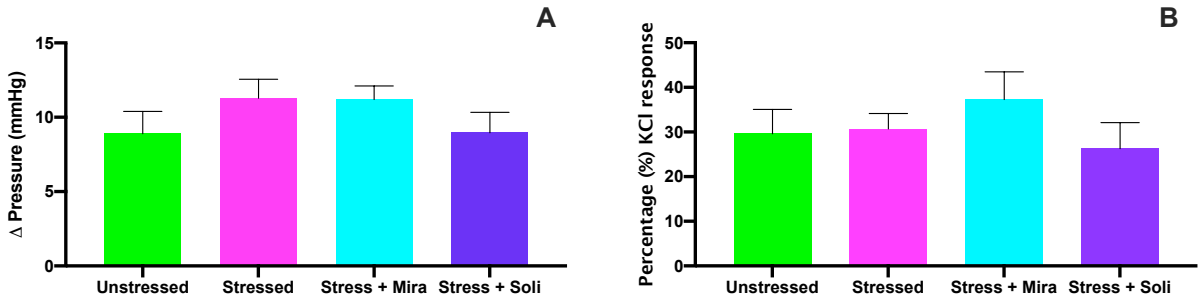

Figure 6.12: Pressure responses to (A) ATP (10 mM) and (B) ATP normalised to KCl response, in isolated bladders from unstressed, stressed, mirabegron (Stress + Mira) or solifenacin (Stress + Soli) treated mice. Datum is represented as mean ± SEM (n = 9) and analysed using an ordinary one-way ANOVA with Tukeys comparison.

Contractile responses to αβmATP (1 mM) were also used to assess purinergic responses and no significant difference was observed between the groups **(Figure 6.13.A)**. Responses were also compared as a percentage of the KCl response, and there was no significant difference between the groups **(Figure 6.13.B)**.

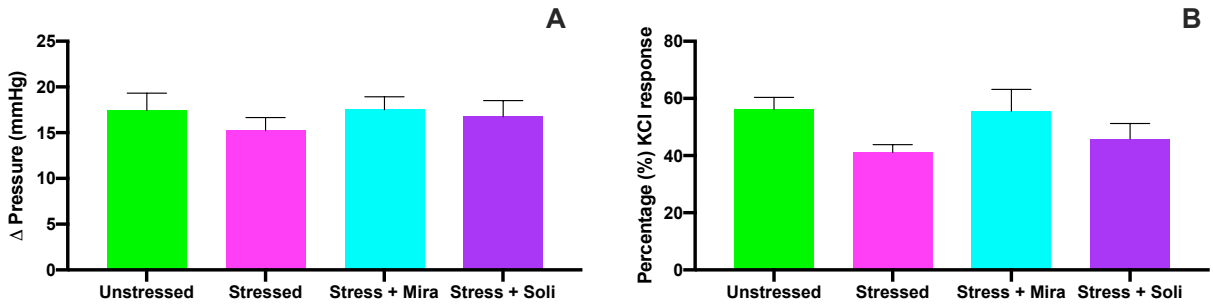

Figure 6.13: Pressure responses to (A) to $\alpha\beta$mATP (1 mM) and (B) as a percentage of the KCl contraction, in unstressed, stressed, mirabegron (Stress + Mira) or solifenacin (Stress + Soli) treated mice. Datum is represented as mean ± SEM (n = 6) and analysed using an ordinary one-way ANOVA with Tukey comparison.

A two-way ANOVA with Tukey multiple comparisons test revealed a significant difference in maximum response to muscarinic agonist carbachol, between bladders from the stressed and unstressed groups. Mirabegron treatment significantly reduced the maximum contractile response compared to the stressed group. Solifenacin treatment did not affect the stress-induced increase in response and the response was still significantly increased compared to the unstressed control ($p = 0.0266$). There was no significant difference in the pEC50 values for carbachol, as highlighted in **TABLE 6.2**. When expressed as a percentage of the KCl response, no significant differences were observed between the groups **(Figure 6.14.B)**. Solifenacin treatment had no effect on the increased contractile response observed after water avoidance stress, while mirabegron reduced the contractile responses to carbachol **(Figure 6.14.A)**.

TABLE 6.2: Whole bladder responses to carbachol in control (unstressed), water avoidance stress (stressed), mirabegron (stress + mira) and solifenacin (stress + soli) treated mice (n=9).

	Unstressed	Stressed	Stress + Mira	Stress + Soli
pEC_{50}	5.55 ± 0.06	5.42 ± 0.06	5.57 ± 0.12	5.41 ± 0.06
Maximal response				
ΔPressure (mmHg)	43.46 ± 1.21	57.45 ± 3.35**	46.97 ± 6.51^{+}	53.23 ± 4.14^
Response (%KCl)	145.19 ± 12.82	158.89 ± 12.92	139.67 ± 14.20	146.67 ± 14.96

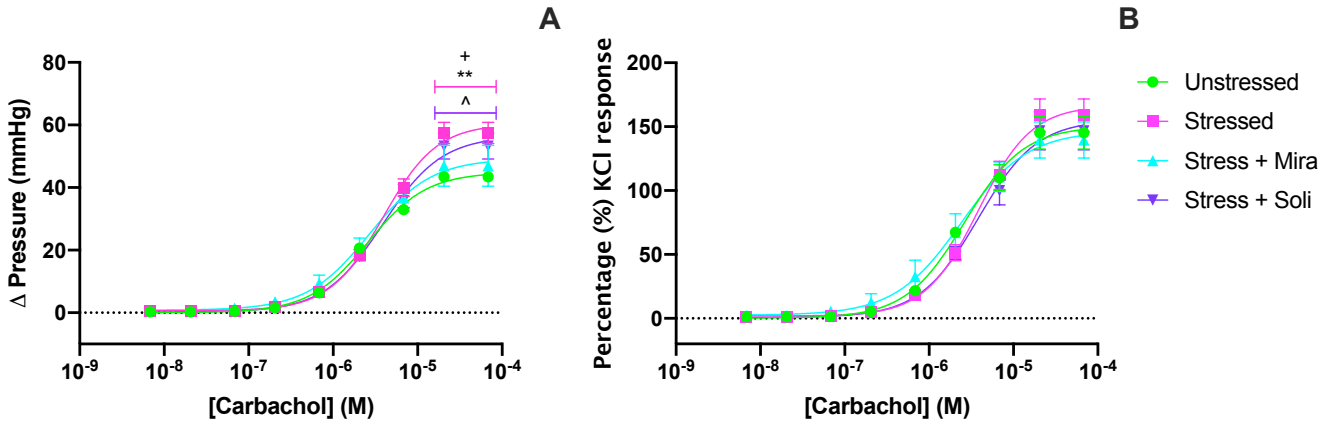

Figure 6.14: Carbachol concentration-response curve, for isolated whole bladders from unstressed, stressed, mirabegron (Stress + Mira) or solifenacin (Stress + Soli) treated mice, recorded as (A) change in intravesical pressure from baseline and (B) change in intravesical pressure as a percentage of the KCl response. Datum is represented as mean ± SEM (n = 9), analysed using non-linear regression and two-way ANOVA with Bonferroni's multiple comparisons test ($p < 0.01$, unstressed vs. stressed) (^$p < 0.05$, unstressed vs. stress + soli) ($^{+}p < 0.05$, stressed vs. stress + mira).**

A concentration-response curve to isoprenaline was performed to assess relaxation of the bladder. There was no significant difference in the pIC_{50} values, however there was a significant difference in maximal pressure response **(TABLE 6.3)**. When expressed as a percentage of the carbachol precontraction the maximum response of bladders from the mirabegron treated group was significantly decreased compared to the unstressed group

(Figure 6.15.B), as displayed in **TABLE 6.3**. Solifenacin had no effect on the relaxation responses of the bladder after psychological stress **(Figure 6.15.A)**.

TABLE 6.3: *Whole bladder responses to isoprenaline in control (unstressed), water avoidance stress (stressed), mirabegron (stress + mira) and solifenacin (stress + soli) treated mice (n=9).*

	Unstressed	Stressed	Stress + Mira	Stress + Soli
pIC_{50}	6.77 ± 0.10	6.83 ± 0.07	6.92 ± 0.10	7.03 ± 0.10
Maximal response				
ΔPressure (mmHg)	-6.07 ± 0.57	-4.84 ± 0.36*	-4.51 ± 0.19~~	-5.04 ± 0.51
Response (% Decrease)	13.26 ± 0.03	32.60 ± 6.81	45.27 ± 8.90	17.69 ± 16.67

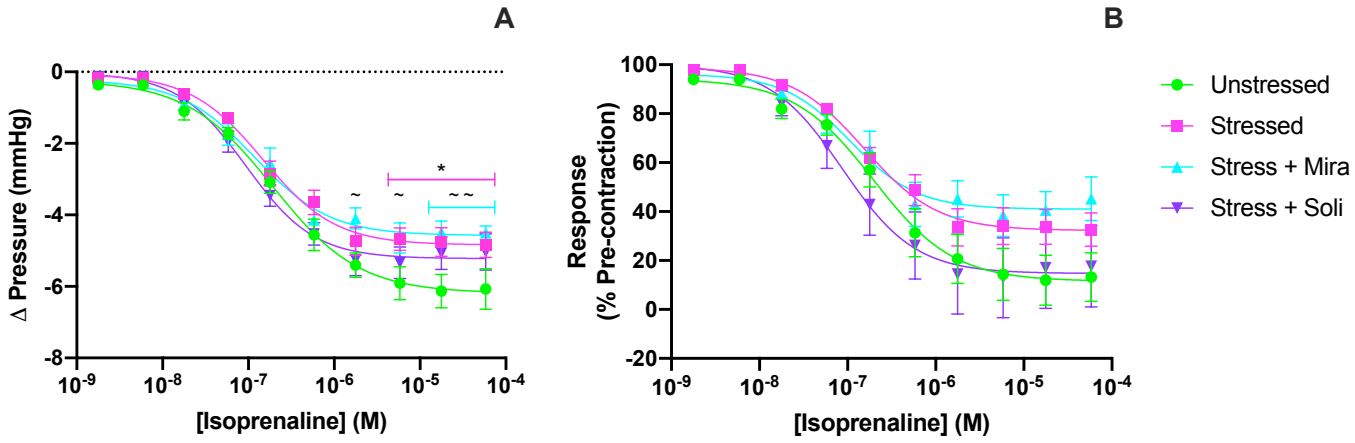

Figure 6.15: Effect of isoprenaline on isolated bladders, from unstressed, stressed, mirabegron (Stress + Mira) or solifenacin (Stress + Soli) treated mice, with datum given as (A) raw data as change in pressure from pre-contraction, (B) change in intravesical pressure as a percentage of the KCl response. Datum is represented as mean ± SEM (n = 9), using two-way ANOVA and non-linear regression (*$p < 0.05$, unstressed vs. stressed) (~$p < 0.05$, ~~$p < 0.01$, unstressed vs. stress + mira).

Spontaneous Contractions

During the stretch-relaxation phase, the frequency of spontaneous activity was increased in bladders from the stressed group, although not significantly **(Figure 6.16.A)**. Mirabegron and Solifenacin treatment decreased frequency of spontaneous activity compared to the stressed group, however this was also not significant. Amplitude of spontaneous activity was unchanged across all groups **(Figure 6.16.B)**.

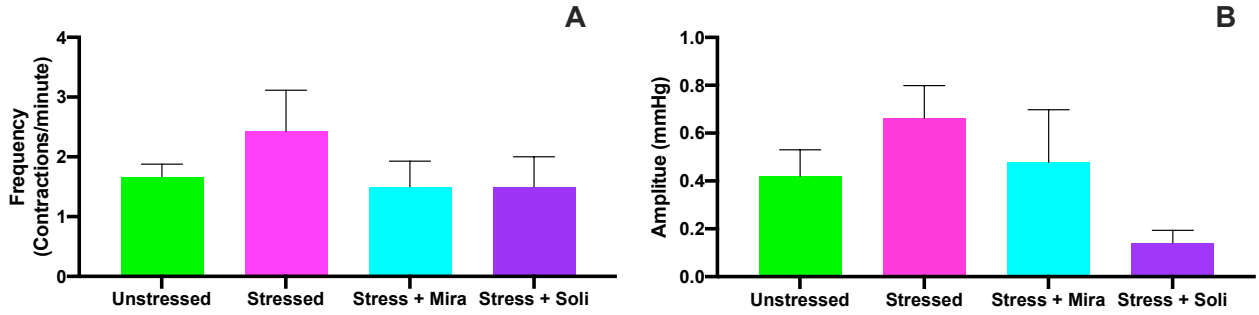

Figure 6.16: Spontaneous contractions during the stretch-relaxation period, in isolated bladders from unstressed, stressed, mirabegron (Stress + Mira) or solifenacin (Stress + Soli) treated mice. (A) Frequency of contractions/minute and (B) Amplitude of spontaneous contractions. Datum is represented as mean ± SEM (n = 6), analysed using one-way ANOVA with Tukey's multiple comparison.

Phasic activity was analysed after a tonic contraction of bladders to the muscarinic agonist carbachol (1 μM). Frequency of phasic activity was significantly increased in the stressed, 7.42 ± 0.68 mmHg ($p < 0.001$) (n=6), and mirabegron treated, 6.33 ± 0.42 mmHg ($p = 0.0027$) (n=6), groups compared to the unstressed group, 3.33 ± 0.21 mmHg (n=6). Solifenacin treatment significantly decreased frequency of spontaneous contractions to 3.66 ± 0.49 mmHg compared to the stressed group ($p = 0.0001$) and was also significantly lower that the mirabegron treated group ($p = 0.0076$) **(Figure 6.17.A)**. The amplitude of phasic contractions was similar across all groups **(Figure 6.17.B)**.

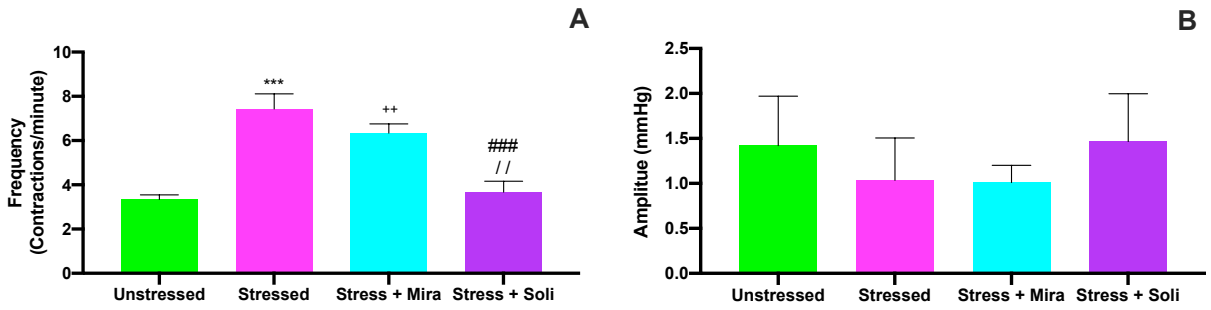

Figure 6.17: Phasic response to tonic contraction of 1 μM carbachol in isolated whole bladders from unstressed, stressed, mirabegron (Stress + Mira) or solifenacin (Stress + Soli) treated mice, following stimulation with carbachol (1 μM) (A) Frequency of phasic contractions and (B) Amplitude of phasic contractions. Datum is represented as mean ± SEM (n = 9), analysed using a one-way ANOVA (*$p < 0.0001$, Unstressed vs. stressed) (++$p < 0.01$, Unstressed vs. stress + mira) (###$p < 0.001$, stressed vs. stress + soli) (//$p < 0.01$, stress + mira vs. stress + soli).**

DISCUSSION

In previous chapters, water avoidance stress was shown to increase voiding frequency and cause increased detrusor muscle contractility. This chapter assessed the ability of mirabegron and solifenacin to resolve the voiding dysfunction and physiological bladder changes caused by stress.

Effects of Mirabegron and Solifenacin on Voiding Behaviour

Mirabegron and solifenacin treatment significantly decreased voiding frequency, compared to animals in the water avoidance stress group. The decreased voiding frequency was accompanied by increased average void size and decreased number of small voids. There was no difference in total voided area, meaning that the mice were still producing the same volume of urine and therefore the changes observed were in voiding phenotype rather than urine production. Both drugs returned voiding behaviour to unstressed control levels, despite continued stress exposure. As discussed previously, mirabegron and solifenacin are common pharmaceutical agents used clinically to treat the symptoms of OAB (Alcantara-Montero, 2016).

Solifenacin and mirabegron have different modes of action, and these differences have been highlighted in several studies. In one study, solifenacin and mirabegron were injected intravenously and bladder and urethral activity were recorded. Solifenacin was found to be more effective in increasing bladder capacity without affecting residual volume (Sugaya et al., 2020). This correlates with another study of solifenacin which used cystometry in rats and also reported that solifenacin increased bladder capacity by 37% at a dose of 0.1 mg/kg intravenously (Suzuki et al., 2005). Studies of mirabegron on rat bladder have shown increased bladder capacity without any effect on voiding pressure or residual volume (Kanai & Andersson, 2010; Sugaya et al., 2020). Another

study using cystometry in conscious rats observed the effects of tolterodine and mirabegron on voiding and non-voiding contractions. The study found that tolterodine decreases voiding contraction while mirabegron did not have this effect and is therefore unlikely to produce urinary retention (Gillespie et al., 2012).

Several studies have examined the effect of mirabegron and solifenacin in a clinical capacity. A review of the safety, efficacy and tolerability of mirabegron has concluded that doses of 50 and 100 mg per day were the most efficient in treating the symptoms of OAB, including micturition frequency and urgency incontinence (Chapple, Cardozo, Nitti, Siddiqui, & Michel, 2014). This is much the same for solifenacin treatment, where clinical studies have shown that patients report improved quality of life, with decreased symptom severity after 5 to 10 mg per day over a 12-week period (Kelleher et al., 2005). Overall, the effects of mirabegron and solifenacin on the urinary bladder would explain why both drugs are able to decrease voiding frequency without affecting total volume.

Effects of Mirabegron and Solifenacin on Corticosterone Release

Repeated psychological stress exposure significantly increased plasma corticosterone levels compared to the unstressed controls, as seen in previous chapters. Interestingly, plasma corticosterone levels were decreased by mirabegron treatment and significantly decreased by solifenacin treatment, compared to the stressed group. Due to these changes, possible sites of action of mirabegron and solifenacin are of particular interest. ACTH is a regulator of steroidogenesis in the adrenal cortex; however, it has been suggested that there are also other local and central mechanisms controlling adrenocortical steroidogenesis, including corticosterone release. Muscarinic receptors are known to regulate numerous central and peripheral functions, and the HPA axis in humans and rodents is reportedly affected by activation or blockade of this receptor

family (Rhodes, Billings, Czambel, & Rubin, 2005). Central muscarinic receptors reportedly modulate corticosterone secretion, with some conflicting evidence of the specific receptor type involved (as reviewed by Thomsen, Sorensen, and Dencker (2018)). A recent study using wild-type, M_2 and M_4 single-knockout (KO) mice reported that administration of a muscarinic agonist stimulates corticosterone release *in vivo* via M_2 muscarinic receptors (Hemrick-Luecke, Bymaster, Evans, Wess, & Felder, 2002). Direct actions of acetylcholine on adrenal cortical cells to simulate steroidogenesis was first reported in 1977 (Rosenfeld, 1955) and later confirmed to rely on activation of muscarinic receptors in several species (Hadjian, Guidicelli, & Chambaz, 1982; Porter, Whitehouse, Taylor, & Nussey, 1988). The presence of cholinergic fibres in the adrenal cortex has also been observed in several species (Edwards & Jones, 1993). This would suggest that the anti-muscarinic, solifenacin may be reducing corticosterone secretion via actions on central or adrenocortical muscarinic receptors.

Studies of micturition function have found the cholinergic, glutamatergic, dopaminergic and GABAergic systems in the central nervous system are all involved in bladder activity (Kanie et al., 2000), for this reason solifenacin may be acting at additional sites. Solifenacin has been found to have low affinity for GABA and dopamine receptors at high concentration and varied affinity for the different muscarinic receptors in humans. Solifenacin has selectivity for M_1, M_2 and M_3 receptors with pK_i values of 7.6, 6.9 and 8.0 respectively (Ikeda et al., 2002), where solifenacin has higher affinity for M_3 receptors over M_2 receptors. M_3 receptors are the minority of muscarinic receptors in bladder smooth muscle, compared to M_2, however studies have found that M_3 plays the predominant role in detrusor muscle contractions of the human bladder (Ehlert, Ostrom, & Sawyer, 1997). M_1 receptors however, are located on pyramidal cells and some axons and nerve terminals (Abrams et al., 2006) in the neocortex, hippocampus and

neostriatum (Volpicelli & Levey, 2004) as well as the forebrain which is known to play a role in the inhibitory mechanisms of the micturition reflex (Yokoyama et al., 2001). Due to the complex neuronal interactions, there may be a link between the effect of solifenacin on M_1 receptors and the decreased corticosterone concentrations that were observed in the present study (Sun, Sun, Zhai, & Dong, 2019).

Research has shown that stress-induced increase in plasma corticosterone in rats is influenced by β-adrenoceptors in the hypothalamic paraventricular nucleus (Morton et al., 1990). Additionally, catecholaminergic nerves are reported to be distributed in the subcapsular regions of the adrenal gland including the zona fasciculata (Charlton, McGadey, Russell, & Neal, 1992; Kleitman & Holzwarth, 1985), with pharmacological approaches indicating that noradrenaline may be an important regulator of glucocorticoid secretion, possibly via β1-adrenoceptors (Walker, Lightly, Clyne, Williams, & Bird, 1991). These provide a plausible mechanism by which mirabegron reduced plasma corticosterone in WAS mice in the current study.

As discussed briefly in the introduction, mirabegron acts specifically on β_3-adrenoceptors which are expressed in a number of different tissues, including the brain (Ursino, Vasina, Raschi, Crema, & De Ponti, 2009). Studies have used the process of reverse transcription-PCR to confirm the presence of β_3-adrenoceptors mRNA in several regions of both the human and rat brain, including hippocampus, amygdala, hypothalamus and cerebral cortex (Rodriguez et al., 1995; Summers, Papaioannou, Harris, & Evans, 1995). Due to the presence of the adrenoceptors in the brain, there has been some interest in targeting these receptor subtypes to treat disorders such as anxiety and depression (Ursino et al., 2009). One study has looked at chronic and acute administration of the β_3-adrenoceptor agonist, CL 316243, and found that acute administration of the agonist increased hypothalamic 5-HT (serotonin) synthesis (Conley, Li, Ivarsson, & Hutson, 2007). Another

β_3-adrenoceptor agonist has also been of interest in treating anxiety and depression (Nisoli, Tonello, Benarese, & Carruba, 1995). A study by Stemmelin et al. (2008) used a number of tests, including the social defeat and forced swim test to measure anxiety and depression related behaviour. The β_3 agonist, SR58611A (amibegron) was given orally to the rats and resulted in anxiolytic and antidepressant behavioural effects (Stemmelin et al., 2008). The study also found that administration of the β_3 agonist resulted in increased synthesis of 5-HT and tryptophan in the cortex, hippocampus, and hypothalamus which correlates with a number of other findings (Claustre et al., 2008). Several links have been made between 5-HT and cortisol/corticosterone release which indicate that 5-HT may influence the peripheral adrenomedullary response to increase cortisol in humans and corticosterone levels in rodents (Cerit, Jans, & Van der Does, 2013; Huang & Herbert, 2005; Murphy & Lesch, 2008).

Overactive bladder symptoms correlate with increased anxiety and depression in patients. While there is some evidence that both mirabegron and solifenacin can reduce the associated symptoms of anxiety and depression in clinical studies (Kelleher et al., 2005; Kinjo et al., 2019), here we observed that oral treatment with solifenacin and mirabegron can reduce the hormonal stress response associated with water avoidance stress.

Effects of Mirabegron and Solifenacin on Mediator Release

Stretch of the urothelium promotes release of mediators, including ACh and ATP, which play an important role in normal bladder function (Yoshida et al., 2006). While ATP concentration levels were unchanged in the present study, ACh release in the serosal fluid was significantly increased in bladders from the stressed animals, as seen in previous chapters, and significantly decreased after mirabegron and solifenacin treatment. It was

briefly described in Chapter 3 that non-neuronal release of ACh has been reported in the bladder urothelium and that this is mediated by CHT1, acetylcholine synthesising enzymes (ChAT and CarAT) as well as an OCT, which is involved in non-vesicular release of ACh from the urothelium (Hanna-Mitchell et al., 2007). While it is unlikely that solifenacin and mirabegron act directly to inhibit non-neuronal ACh release from the urothelium, there is some evidence from an in vitro study that shows mirabegron has weak inhibition of OCT1 in mouse kidney-derived S2 cells, with an IC_{50} of 47.2 μM (unpublished data reported by Groen-Wijnberg et al. (2017)). Interestingly, this concentration is 175-fold higher than the mean maximum plasma concentration in healthy subjects after the administration of mirabegron at 50mg once a day, the highest approved dose (Krauwinkel et al., 2012). There is, however, no evidence that solifenacin has any effect on these transporters.

Multiple studies have identified that the urothelium expresses a range of muscarinic receptors, including in the mouse (M_1 - M_5) and human (M_1, M_2, M_3 and M_5) bladder (Mansfield et al., 2005; Zarghooni et al., 2007). Along with muscarinic receptors, beta-adrenoceptors have also been identified in human (β_1 – β_3) and rat (β_1 – β_3) bladder urothelium (Kullmann et al., 2011; Otsuka, Shinbo, Matsumoto, Kurita, & Ozono, 2008). The studies have shown that these receptors all contribute to normal physiology of the bladder, however it is still unknown whether their function may affect non-neuronal ACh release. It may be possible that the effect on ACh release was due to the decreased plasma corticosterone levels by mirabegron and solifenacin, rather than a direct effect at urothelial receptors as ACh release from the urothelium may act via a negative feedback mechanism, inhibiting further release of ACh (Hanna-Mitchell et al., 2007).

Effects of Mirabegron and Solifenacin on Bladder Physiology

While voiding frequency was reduced by mirabegron and solifenacin treatment, the increase in bladder contractility observed following WAS was not altered in the solifenacin and mirabegron treated animal groups. This would suggest that the increase in contractility does not play a causal role in the voiding dysfunction observed with stress and may instead be a local compensatory mechanism in response to stress. M_3 are the predominant muscarinic receptors involved in mediating contraction in the mouse urinary bladder (Choppin & Eglen, 2001), and solifenacin is a muscarinic antagonist, acting on M_3 receptors to decrease contraction. Studies of pathogenic bladder dysfunction in humans however have found that both M_2 and M_3 receptors may be mediating bladder contractile responses in pathological states, i.e., M_2 receptors play a greater role in pathological states. Darifenacin, a M_3 receptor antagonist, did not affect bladder contractions until both M_2 and M_3 receptors were inhibited (Ruggieri & Braverman, 2006). In the present study solifenacin may be acting to block the predominant M_3 receptor subtypes, but M_2 receptors may have taken over contractile role, affecting bladder contractile responses, while not contributing to an increase voiding phenotype.

An *in vitro* study on mouse urinary bladder reported that solifenacin does not alter the maximal response to carbachol (Salcedo et al., 2009), unlike darifenacin. Similarly, solifenacin increases bladder capacity without affecting maximum micturition pressure (Liu et al., 2017; Suzuki et al., 2005) in rodents *in vivo*. In Chapter 4, we postulated that the enhanced contractile response caused by repeated WAS may involve calcium-sensitization through the Rho-kinase pathway. It is therefore not surprising that solifenacin did not affect this change in contractility, as it is most likely independent of receptor type.

β-adrenoceptor relaxation of human detrusor is mediated predominately by β_3-adrenoceptors (Wuest et al., 2009), whilst most studies have reported involvement of both β_2 and β_3-adrenoceptors in rodents (Takeda et al., 2003). A study has measured β-adrenoceptor subtype expression by exposing KCl pre-contracted detrusor muscle strips of wild type and β_2 receptor KO mice, to β-adrenoceptor agonist, isoprenaline and the β_3-agonist, CL 316,243. The study found that the predominant receptor responsible for relaxation was the β_2-adrenoceptor, while β_3-adrenoceptors can also mediate relaxation (Propping, Lorenz, Michel, Wirth, & Ravens, 2016).

The present study found that maximal relaxation of bladders to isoprenaline was reduced in the mirabegron treated animals compared to the unstressed control animals. In the study mentioned above, in the absence of β_2 receptors, isoprenaline concentrations required for half the maximum relaxation were approximately 90-fold higher (Propping et al., 2016). Studies of mouse urethra have also demonstrated similar findings. A study by Alexandre et al. (2016) found that isoprenaline produced a biphasic pattern of relaxation in isolated mouse urethra which turned monophasic at high concentrations with β_3-adrenoceptor antagonist L-748,337 causing a contraction at the end of the curve. Mirabegron on the other hand produced full relaxation responses in mouse urethra, up to 1μM (Alexandre et al., 2016), whereas isoprenaline at high concentrations interacts with α_1-adrenoceptors, behaving as a weak partial agonist (Trendelenburg, 1974). This changed when urethra strips were pre-contracted with phenylephrine. The relaxation curve after pre-contraction was shallow in the mirabegron treated group and L-748,337 was able to antagonise the effects of mirabegron. This indicated that different mechanisms might be responsible for the relaxations produced at low or high concentrations of mirabegron (Alexandre et al., 2016). While these experiments were performed in the urethra, the results may be translatable to the bladder.

There is also a possibility of desensitisation in β-adrenoceptors. Rat bladder strips following 6-hour pre-treatment with 1 μM isoprenaline or mirabegron, amongst other β agonists had reduced maximum relaxation responses. This desensitization was prominent for β_2-adrenoceptor mediated relaxation but much weaker for β_3, however, the acute agonist pre-treatment may account for this (Michel, 2014). In the current study mice were treated with mirabegron at a clinically relevant dose for 10-days, following which the maximal relaxation response was reduced. While this may reflect desensitization of β_2 and/or β_3 adrenoceptors in the mouse bladder, it did not affect the efficacy of this drug in terms of its ability to manage the voiding dysfunction caused by WAS.

The frequency of the phasic response induced by muscarinic stimulation was significantly enhanced by stress and reduced by solifenacin treatment. Afferent nerve terminals are located adjacent to the bladder urothelium and stimulation of urothelial muscarinic receptors may contribute to activation of afferent nerves via non-neuronal release of ATP, causing urinary urgency (Yoshida et al., 2009). Phasic activity in the bladder wall may also arise from spontaneous increases in Ca^{2+} which are always preceded by action potentials. The action potentials and calcium increase occur along the boundary of the smooth muscle and then move through the gap junctions (Hashitani, Fukuta, Takano, Klemm, & Suzuki, 2001). Non-voiding activity has been found to be sensitive to muscarinic antagonists and β_3-adrenoceptor agonists. Cytometry was performed in conscious rats with replicated partial bladder outflow obstruction were given intravenous mirabegron and tolterodine (Gillespie et al., 2012). The study found that as the fill progressed, both drugs reduced large non-voiding contractions, mirabegron only affected frequency but tolterodine affected both frequency and amplitude (Gillespie et al., 2012). There is also evidence that mirabegron may attenuate

micro contractions by acting on the β_3 adrenoceptors of afferent nerves. Data has shown that mirabegron in rats inhibits Aδ-fibre mechano-sensitive afferent activity, meaning that there is decreased afferent signalling from the periphery to the brain (Aizawa et al., 2012). Decreased spontaneous activity may be another therapeutic effect of solifenacin treatment while decreased afferent signalling may be another therapeutic effect of mirabegron thereby leading to decreased urgency to void and decreased urinary frequency in the treated mice.

There is a possibility that both mirabegron and solifenacin had an effect on other locations where autonomic receptors are localised. For example, within the urinary system, β_3 adrenoceptors have also been located in the nephron segments of the kidney and when stimulated with the selective agonist BRL37344, urine concentration increased while urine excretion decreased (Procino et al., 2016). Evidence also suggests that some neurons within the pelvic ganglion in rats express β_3 adrenoceptors, and when stimulated, are involved with regulation of efferent outflow and sensation (Eastham, Stephenson, Korstanje, & Gillespie, 2015). In the case of solifenacin, M_3 receptors have been identified in numerous areas other than the detrusor muscle. Previously M_1, M_2 and M_4 muscarinic receptors have been identified on sympathetic nerve endings, regulating noradrenaline release and on parasympathetic nerve endings, regulating ACh release (Chess-Williams, 2002). While these muscarinic receptor subtypes are less affected by solifenacin, there is evidence that in diseased states, there is upregulation of M_3 receptors at M_1 receptor sites (Somogyi et al., 2003). As seen in the current and previous chapters, psychological stress induces physiological changes within the bladder which in turn leads to voiding dysfunction. While we have examined the bladder changes specifically here, there may be other physiological changes occurring which could be remedied by solifenacin and mirabegron treatment.

CONCLUSION

Treatment with the muscarinic antagonist, solifenacin and β adrenoceptor agonist, mirabegron, decreased the effects of water avoidance stress on voiding behaviour to unstressed control levels. Both therapeutics also reduced changes in ACh urothelial mediator release and plasma corticosterone levels that were observed following stress. These results indicate that management of bladder dysfunction caused by psychological stress may benefit from the addition of an antimuscarinic such as solifenacin or the β_3-adrenoceptor agonist, mirabegron.

CHAPTER 7: GENERAL DISCUSSION

The purpose of these studies was to understand the effects of psychological stress on overall physiological bladder function and which currently used treatments may aid in combating this dysfunction. There is abundant clinical evidence that psychological stress is associated with bladder dysfunction (Lai, 2015), however the underlying local mechanisms involved in this dysfunction are still generally unknown. There is also recent evidence that suggests that diagnosis of bladder pathologies should include measurement of serum and urine concentrations of stress-related hormones such as cortisol and CRF, in order to tailor treatment of overactive bladder to the patient, highlighting the significance of psychological stress in the development of bladder disorders (Peyronnet, Chapple, & Cornu, 2019).

Here, a body of work was described which initially assessed the impacts of different types of psychological stress on bladder function. Social defeat and witness trauma stress (Chapter 3) and water avoidance stress (Chapter 4, Part 1), surprisingly, produced vastly different bladder pathologies, observed mainly as changes in voiding frequency. Water avoidance stress produced an overactive bladder phenotype, whereas social defeat produced a urinary retention pattern. Given the numerous clinical reports of increased urinary frequency due to stress, we used the water avoidance model to assess the effect of a recovery period (Chapter 4, Part 2) and pharmacological intervention (Chapter 5 and 6) on these changes.

While the general findings of this thesis are that psychological stress increases urinary frequency, as well as overall bladder contractility, and that these changes can be remedied, at least in part, by stress-free recovery time and pharmacological treatments, there is some dissemblance in the chapters which will be discussed here in the General Discussion.

THE DIFFERENT MODELS OF PSYCHOLOGICAL STRESS

Between the social defeat/witness trauma model and the water avoidance model there were various differences in voiding behaviour and overall physiological bladder mechanisms. It is therefore worth discussing the differences between the models.

To begin, social defeat and witness trauma stress are models of social stress. Unlike previous studies, which have only focused on the social defeat aspect, our study allowed for the observation of both emotional and physical stress, as well as the aspect of a social support (Li et al., 2018). Water avoidance stress however replicates an environmental stress model (Yamamoto et al., 2012). As stated above, both studies produced phenotypes of voiding dysfunction, however, social defeat stress appeared to lead to urinary retention while water avoidance stress led to bladder overactivity. The increased purinergic response in bladders from the social defeat animals appears to lead to a retention phenotype according to our study and others (Mann, 2015; West et al., 2020). There could be some argument however that the term 'retention' does not quite fit this model, and instead the effect could be due to more efficient nerve evoked contractions and therefore more efficient voiding. These pathological differences observed could be due to several factors including the model of psychological stress itself and its duration, the changes in underlying bladder mechanisms or the differences in sex of animals used in the models.

The majority of studies using a model of social defeat have used male rodents, due to the challenge in implementing the model in female rodents (Harris et al., 2018). Our model of social defeat produced a urinary frequency phenotype similar to what would be seen in urinary retention, corresponding with other studies of this model (Mann, 2015; Wood et al., 2009). There was one study however which found that social stress in 6-week-old male mice, lead to increased urinary frequency evident after analysis of bladder afferent

nerve activity (Mingin et al., 2015). Currently there are no studies that have observed the effect of social defeat on the voiding behaviour of female mice. This is reported to be mainly due to the complexity of the model, as male and female mice will typically not attack intruder females (Harris et al., 2018). Research groups have attempted to mediate this by using a model of maternal aggression (Bourke & Neigh, 2012) or social instability (Haller, Fuchs, Halasz, & Makara, 1999). Neither of these models, however, have been used to assess bladder function.

In contrast to the social defeat model, water avoidance stress has been undertaken in both male and female rodents, with varying results. Interestingly, one study of water avoidance stress found that male mice presented with a decrease in voiding frequency and increase average voided volumes (McGonagle et al., 2012), similar to that seen in social defeat stress. Another study however contradicts this finding, with increased urinary frequency observed in male mice (Yamamoto et al., 2012). Water avoidance stress, has however, been used to study several aspects of bladder function in female mice, with most studies concluding that the model leads to increased urinary frequency (Lee et al., 2015; Matos, 2017). Sex differences between models of psychological stress have not been directly studied with regard to bladder function, however several studies have looked at this difference with regard to other pathologies reporting sex differences in neural and behavioural responses to psychological stress (Gruene, Flick, Stefano, Shea, & Shansky, 2015; Hodes et al., 2015; Lebron-Milad & Milad, 2012; Shansky et al., 2010). Sex differences in relation to bladder dysfunction are clinically relevant however since both anxiety related disorders (Breslau et al., 1995) as well as the symptoms of overactive bladder syndrome are more prevalent in woman than men (Temml, Heidler, Ponholzer, & Madersbacher, 2005).

PHYSIOLOGICAL BLADDER CHANGES

It should be noted here that there were some differences in bladder responses between the original water avoidance stress study (Chapter 4, Part 1) and the other studies in this thesis where the model was used (Chapter 4, Part 2 and Chapter 5 and 6). The magnitude of all bladder responses in the whole bladder preparation studies was generally increased from Chapter 4, Part 2 onwards, and the only aspect of the project which may have contributed to this was the time between experimental studies. However, each experimental study was performed with separate groups of age-matched unstressed controls and separate WAS animals, and so we are confident that despite some changes in the magnitude of the responses, the difference observed between the groups are genuine.

It was clear throughout the various studies that mice were experiencing psychological stress, due to the increase in plasma corticosterone levels seen in the stressed animals. In terms of physiological bladder changes, an increase in the purinergic system was observed in the social defeat paradigm, while water avoidance stress caused a general increase in bladder contractility in stressed groups, consistent across chapters. These common changes seen across chapters will be discussed here.

For many years the bladder urothelium was considered to act as a passive barrier, however, studies have shown that the urothelium has specialised sensory and transduction properties (Merrill et al., 2016). The release of urothelial mediators, including ATP and ACh has been shown to change depending on urothelial stretch and chemical stimuli, and may affect sensory signalling and overall bladder function (Apodaca et al., 2007). Fry and Vahabi (2016) undertook a review of studies using the whole bladder, isolated sections of the bladder wall, detrusor-free mucosa and isolated urothelial cells, and concluded that all studies reported release of ATP, ACh,

prostaglandins and NO, when physical or chemical stress was applied. As all of these preparations released these mediators, it would be correct to assume that the source is the urothelium (Fry & Vahabi, 2016). These studies highlight the complex interaction between the detrusor and urothelium and support the belief that mediators are able to move throughout the layers of the bladder (Fry & Vahabi, 2016).

Interestingly, across all chapters of this thesis there was a trend towards increased release of ACh into the intraluminal fluid (social defeat and witness study) and serosal fluid (WAS studies).

In urothelial cells, choline uptake transporters, as well as the enzymes for ACh synthesis, have been identified (Hanna-Mitchell et al., 2007). Typically, changes in urothelial release of ACh has not been implicated in diseased states, only with age (Yoshida et al., 2006), while stretch-evoked release of ATP has been linked to a number of conditions of bladder dysfunction (Birder & de Groat, 2007). Studies of aged bladder tissues from both animals and human have found that ATP release is increased with age (Daly et al., 2014; Yoshida, Miyamae, Iwashita, Otani, & Inadome, 2004), while there are conflicting opinions whether ACh cholinergic transmission decreases (Daly et al., 2014) or is enhanced with age (McDermott et al., 2013). Bladder biopsies from OAB patients also revealed that ATP levels were elevated (Munoz, Smith, Boone, & Somogyi, 2011).

As stated in relevant chapters, non-neuronal release of ACh is not completely understood, but could have a number of consequences in bladder physiology. As stated above, after release, urothelial mediators typically go on to interact with cell in several layers of the bladder (Fry & Vahabi, 2016) **(Figure 7.1)**. This suggests that non-neuronal release of ACh may have both autocrine and paracrine signalling roles, potentially stimulating further release of ATP, NO or prostanoids from urothelial cells, or acting back on its own receptors (Wessler & Kirkpatrick, 2008). There is also possibility that non-neuronal ACh

may act on the detrusor muscle directly. There has been convincing evidence that the detrusor muscle and urothelium have a complex interaction. Studies of detrusor and attached urothelial mucosa have found that when detrusor is denuded, spontaneous activity is reduced (Kanai et al., 2007; Sui et al., 2008). As we saw throughout the stress studies, both types of psychological stress increased phasic contractile activity, which is known to play a role in several bladder pathologies (Meng et al., 2008). It is therefore possible that non-neuronal release of ACh contributes to this increase in spontaneous activity by stimulating the release of ATP, the mediator which has been implicated in increased spontaneous activity (Kushida & Fry, 2016). There is evidence that non-neuronal released ACh may also be acting on afferent nerve fibres via both nicotinic and muscarinic ACh receptors (Apodaca et al., 2007). There is conflicting evidence however, as to the effect ACh has on these receptors. Evidence suggests that stimulation of muscarinic receptors can depress sensory transduction via afferent nerves (Daly et al., 2010; Kim, Yoshimura, Masuda, de Miguel, & Chancellor, 2005), while studies of nicotinic receptors indicate that stimulation may enhance bladder afferent nerve activity originating at afferent receptors near the urothelium (Yu, Daugherty, & de Groat, 2016). If this were the case, stimulation of nicotinic receptors would lead to alterations in bladder sensation, which may be involved in the increased urinary frequency that was observed in water avoidance stress animals.

A summary of urothelial ACh release and subsequent actions of the mediators is given below in **Figure 7.1**.

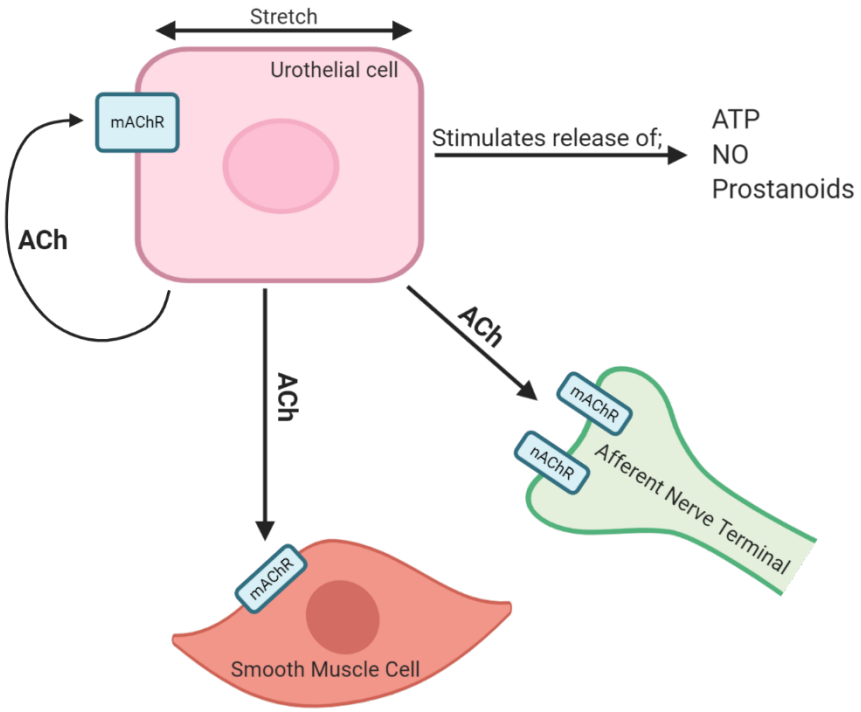

Figure 7.1: Interactions of mediators from urothelium after urothelial cell stretch during bladder filling. Interactions occur between urothelial cells as well as afferent and efferent nerves, smooth muscle cells and interstitial cells. Acetylcholine (ACh), Adenosine triphosphate (ATP), Muscarinic receptors (mAChR), Nicotinic receptors (nAChR), Nitric oxide (NO). (Created with BioRender.com by the author).

As discussed above, ATP release was unchanged in bladders across all groups in all chapters, which was unexpected due to the link between urothelial ATP release and bladder disorders (Birder & de Groat, 2007). Studies of ATP release during bladder filling have indicated that urothelial ATP release occurs via exocytosis during the early stages of bladder filling (Nakagomi et al., 2016). Another study of whole rat bladder measured mediator release at low and high distensions and found that at both pressures, ATP was released in a similar amount (Bravo et al., 2017). This finding was supported by another study which looked at luminal ATP release in women with detrusor overactivity. The study found that ATP concentrations were only increased in the early filling stage (200 mL) of bladder distension as opposed to the later stage of filling (400 mL) (Cheng et al.,

2014). This may explain why we did not see a change in ATP release, since we collected intraluminal and serosal fluid at the later stage of filling (20mmHg) from the mouse bladders.

Overall, enhanced contractility following water avoidance stress does not completely explain why stressed mice experience increased urinary frequency, since stronger contractility could be thought to induce more efficient voiding. This is particularly relevant in the recovery group, which showed decreased overall bladder contractility, possibly due to increased compliance (as discussed in Chapter 4, Part 2), but still exhibited increased urinary frequency. This data suggests that there is a possible change occurring in afferent nerves, to trigger voiding at lower volumes. Work by others in our group, currently unpublished, shows that at low physiological pressures, afferent nerve activity is enhanced by water avoidance stress **(Figure 7.2)**. This indicates that stressed mice are experiencing enhanced sensory activity and subsequent voiding at lower physiological pressure and volumes. The changes in bladder contractility may therefore by a compensatory mechanism rather than a causal factor.

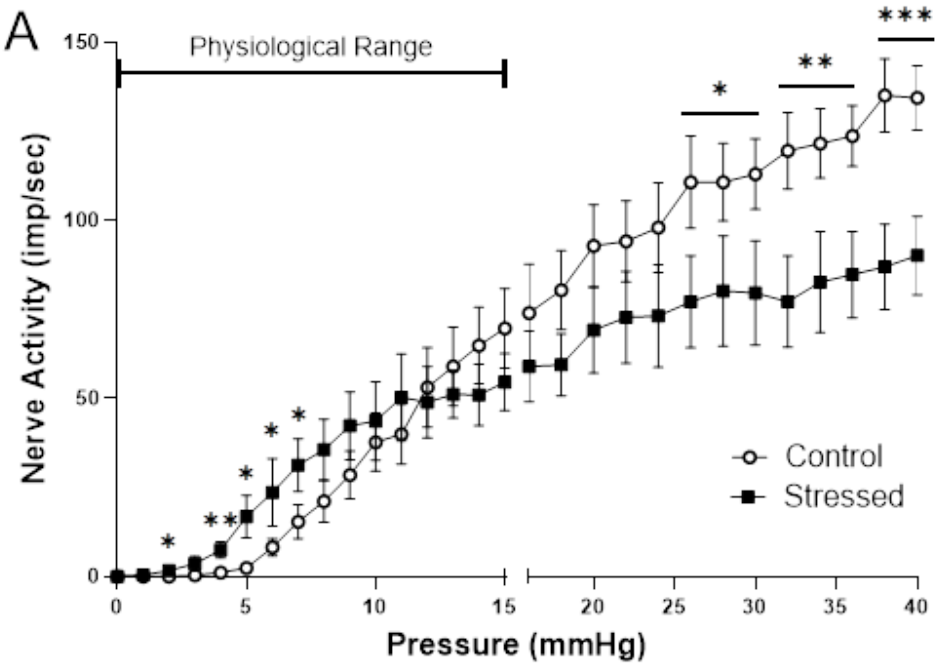

Figure 7.2: Afferent nerve recording during filling of bladder, from mice after 10-day water avoidance stress (Stressed) and Unstressed (Control) groups. Physiological range is indicated above graph and bladders are filled to a pressure of 40 mmHg. (Unpublished data, reproduced with permission from Kylie Mills, 2020).

TREATMENT OF PSYCHOLOGICAL STRESS AND BLADDER DYSFUNCTION

The final question of this thesis lies in what the most suitable treatment would be to combat bladder dysfunction caused by psychological stress. In Chapter 5 and Chapter 6, we found that sertraline, an anxiolytic drug, as well as mirabegron and solifenacin were successful in reducing the overall increase in bladder contractility seen in the stressed groups. To distinguish, between the most appropriate treatment, the interactions of each drug and subsequent adverse effects should be considered.

Sertraline is an SSRI used in the management of anxiety and depression but unlike other SSRI's, it has very few adverse effects. The potential use of sertraline in treating overactive bladder stems from studies which have found that depletion of serotonin within the CNS leads to increased urinary frequency and detrusor overactivity (Chiba et

al., 2016). The role of sertraline is predominantly to increase serotonin availability within the brain which may then act on serotonergic neurons involved in descending inhibition of the bladder, including the Onuf nuclei and the lumbosacral autonomic nuclei, which innervate urethral sphincters (Andersson & Pehrson, 2003). As discussed, sertraline acts predominately in the CNS, but there may be potential local bladder 'flow on' effects of sertraline (Konthapakdee et al., 2019). 5-HT receptors are located throughout the bladder and depending on the location, play different roles in bladder physiology. Interestingly, stimulation of 5-HT with serotonin has been shown to increase bladder afferent nerve activity and significantly increase detrusor muscle contractility (Konthapakdee et al., 2019). This is supported by other findings that selective 5-HT receptor agonists cause contraction of bladder strips (Hattori, Lluel, Rouget, Rekik, & Yoshiyama, 2017). These findings would seem to contradict our studies of sertraline here, as afferent nerve firing would most likely be leading to early voiding. However, early studies also observed that stimulation of 5-HT receptors suppresses the processing of afferent input from the bladder, resulting in inhibition of preganglionic neurons and excitation of sympathetic neurons, leading to decreased contractility and increased urine storage (De Groat & Ryall, 1967; Espey et al., 1992). There is evidence, however, that sertraline can desensitise 5-HT receptors after chronic administration in the rat cortex (Sanders-Bush, Breeding, Knoth, & Tsutsumi, 1989). This is supported by a more recent study which found that mild administration of sertraline was effective in reducing depression-like behaviour, however towards the end of the treatment phase, there was a rapid onset of depressive behaviour, indicating desensitisation of 5-HT receptors within the brain (Mukherjee, Sen, Biswas, Barman, & Tripathi, 2015). Clinically, patients have reported moderate-to-severe depression while on a long-term antidepressant treatment

regime, which may indicate desensitisation in clinical cases (Cartwright, Gibson, Read, Cowan, & Dehar, 2016).

Mirabegron and solifenacin are drugs that act directly on the bladder, increasing relaxation as a β-adrenoceptor agonist and decreasing contraction, amongst other actions, as an M_3 muscarinic antagonist respectively (Abrams et al., 2015). Unlike sertraline, mirabegron and solifenacin are not thought to directly target the CNS to reduce anxiety and depression but rather target the bladder directly to combat overactive bladder symptoms. Studies have found a link however, between targeting bladder symptoms and improving quality of life with solifenacin treatment (Kelleher et al., 2005), and decreasing anxiety symptoms after mirabegron treatment (Kinjo et al., 2019). There is evidence that these therapeutics may be targeting other areas of the body as well as the bladder.

Mirabegron, for instance, specifically targets β_3 receptors which are present within the brain, heart and throughout the gastrointestinal tract as well as in the bladder (Dehvari, da Silva Junior, Bengtsson, & Hutchinson, 2018). One study has observed that mirabegron increases the uptake of glucose into brown adipose tissue, however, at the dose of 200mg per day, there was adverse cardiac effects within the cohort (Cypess et al., 2015). The role of β_3 adrenoceptors within the heart has long been debated, but it is known that heart failure is induced by sustained activation of $\beta_{1/2}$ adrenoceptors, due to increased catecholamine release from the sympathetic nerves (Bernstein, Fajardo, & Zhao, 2011). This causes desensitisation and internalisation of the receptors and loss of contractile function and cardiac remodelling (Dehvari et al., 2018). Interestingly, the expression of β_3 adrenoceptors increases in rodent heart after prolonged stimulation by catecholamines (Germack & Dickenson, 2006) and during cardiac disease in human myocardium (Moniotte et al., 2001). This may be a protective mechanism for the heart

under myocardial stress and stimulation of these receptors in mice has shown no change in overall cardiac function (Belge et al., 2014). This indicates that targeting of β_3 adrenoceptors may be beneficial in treating heart failure, as these receptors are not desensitised after prolonged agonist exposure (Dehvari et al., 2018). A clinical trial observing left ventricular ejection fractions (LVEF) found that mirabegron significantly improved LVEF compared with patients given a placebo (Bundgaard et al., 2017). In terms of the bladder, β_3 adrenoceptors have been found to not be internalised or degraded like cardiac $\beta_{1/2}$ adrenoceptors (Dehvari et al., 2018), but responses may be affected by downregulation of other components of the signalling pathway, at the adenylyl cyclase level (Michel-Reher & Michel, 2013). Clinically, this means that the features of β_3 adrenoceptors allow for long term pharmacological stimulation with limited adverse effects or risk of desensitisation (Schena & Caplan, 2019).

Solifenacin may have several tissue targets due to the wide range of locations where M_3 receptors are present. Studies have shown that M_1 and M_3 receptors are present in the salivary gland (Culp, Luo, Richardson, Watson, & Latchney, 1996), while only M_3 is present in the parotid glands (Watson et al., 1996). Salivation is therefore predominately mediated by M_3 receptors which is illustrated in preclinical trials which have demonstrated that selective antagonism of M_3 receptors inhibits salivary response to carbachol (Ikeda et al., 2002). Clinical studies have found that M_3-selective drugs, such as solifenacin appear to reduce salivation, leading to dry mouth and discontinuation of treatment (Chapple et al., 2005). Within the gut, M_2 receptors outnumber M_3 receptors, but M_3 still plays a prominent role in cholinergic stimulation of gastrointestinal activity (Matsui et al., 2002), in a similar way that M_3 receptors play a prominent role in detrusor contraction within the bladder (Abrams et al., 2006). Interestingly, serotonergic receptors have also been identified within the gastrointestinal tract, where 5-HT_4

mediates excitatory effects (Gershon, 2003), which explains why M_3 KO mice, in another study, had no explicit gastrointestinal problems (Matsui et al., 2000). There is also evidence for expression of all five muscarinic subtypes in the brain, however, M_3 receptor expression is low in the brain (Volpicelli & Levey, 2004). Similarly, all five muscarinic receptor subtypes are present within the eye, however, in contrast to the brain, M_3 is the predominant receptor. Although ocular events have been reported to occur during muscarinic receptor antagonism, the selective M_3 antagonist, darifenacin, has been reported to cause no greater blurred vision compared to the less selective agent, oxybutynin (Zinner, Tuttle, & Marks, 2005).

When choosing a treatment for psychological stress and increased urinary frequency, the primary site of action of the drug would have to be taken into consideration. As stated above, sertraline primarily acts centrally before having downstream effects on the bladder, which is evident in the urinary frequency data, where frequency was not reduced back to unstressed control levels, as was observed after mirabegron and solifenacin treatment. It is also evident that all three treatments reduced the hormonal stress response, although not significantly so for mirabegron. It can therefore be concluded that environmental psychological stress induces urinary frequency and that the best available treatment for both conditions is the anti-muscarinic solifenacin. In terms of managing voiding dysfunction alone, mirabegron and solifenacin were equally effective and superior to sertraline.

STRENGTHS AND LIMITATIONS

The experiments carried out in the Social Defeat model as well as the Water Avoidance Model demonstrated that both social and environmental stress impact psychological wellbeing as well as overall bladder function. The Social Defeat model, carried out on male mice, caused urinary retention patterns and while this is a disorder that is understood to be prevalent within the community, as a research group we were interested in the pathophysiology of incontinence specifically which is why, after analysis of both models, the water avoidance model was chosen. It would be interesting, however, to understand more thoroughly the pathophysiology of psychological stress induced urinary retention and this model would be beneficial in that area of future study.

The limitation of only being able to complete this Social Defeat model on male mice also impacted the overall thesis design as it would have been valuable to have the same sex mice use throughout the experimental studies, for continuity. As discussed previously however, there is still discussion to be had around the viability of a female mouse social defeat study, as typically, male mice are used for their territorial nature.

The overall finding of the water avoidance drug model was that both Solifenacin and Mirabegron were able to reduce both psychological stress indicators (corticosterone) as well as the incontinent voiding pattern back to baseline urinary data. Sertraline was also efficient in reducing psychological stress levels, however, urinary symptoms of incontinence were not completely recovered with treatment. Now that experimental work is complete and knowing what we know now, it may be useful to think about prolonging the Sertraline treatment. Typically, the severity of anxiety and depression symptoms occur in 'waves' and patients will be on treatment for an extended period of time to manage these symptoms. If the water avoidance model was to mimic typically what occurs in a human population, I would suggest a model that extends for several

more weeks where the mice undergo intermittent periods of variant stress rather than the 10 continuous days that we focused on. This may better reflect a real time stress scenario where the bladder may be allowed to recover which may provide interesting and helpful information regarding dosage and medication regimes required for patients suffering from both psychological stress and bladder dysfunction.

Whole bladder experiments conducted were able to give interesting insight as to the pathophysiology behind that bladder dysfunction we witnessed in the stressed mice. Due to changes seen in mediator release and electrical field stimulation, it may be useful to study this change in an afferent nerve activity. In future studies it may also be interesting to look into bladder histology which would allow us to understand if the psychological stress or drugs used during this period, produced any long-term change within the bladder wall leading to dysfunction.

CONCLUDING REMARKS

As with most scientific research, work in this thesis has generated a number of questions and interesting topics for future investigations. Firstly, the results presented here support a role for increased afferent nerve signalling and overall bladder contractility as a result of psychological stress.

Investigations into mediator release at lower levels of bladder distension would help us understand the role that urothelial ATP and ACh are playing in this type of bladder dysfunction. Furthermore, investigation into the role that ACh plays in bladder dysfunction would provide a link between the increased release of this mediator and its effect in psychological stress-induced bladder dysfunction.

Very little information is available on the long-term effects of psychological stress on bladder dysfunction. Clinically this would be relevant as psychological stress presents as anxiety and depression in a human population and these are ongoing disorders known to repeat over a long period of time. It would therefore be beneficial to analyse bladder function over a longer period of time, where psychological stress is introduced periodically and is more representative of the life-long periodic stress experienced in humans.

In conclusion, this thesis has found that different forms of psychological stress cause variations of bladder dysfunction. Water avoidance stress was associated with detrusor hypercontractility, resulting in bladder overactivity. The impact of water avoidance stress on voiding behaviour was reduced equally by mirabegron and solifenacin treatment and both drugs were superior to the selective serotonin reuptake inhibitor, sertraline.

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